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Sperm quality of male redclaw crayfish (*Cherax quadricarinatus*): Insights from conventional and advanced sperm diagnostic tools, methods of spermatophore collection, and broodstock nutrition

Thesis submitted by

Jon Irish Legaspi Aquino, MSc, RFP

November 2023

In fulfilment of the requirements for Doctor of Philosophy (Science)

College of Public Health, Medical, and Veterinary Sciences

James Cook University, Townsville, Australia

Acknowledgments

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Chapter	Title	Nature of contribution of co-authors
1	General introduction	**Jon I. L. Aquino**: Conceptualization, writing - original draft **Leo Nankervis**: Review & editing **John Cavalieri**: Review & editing, supervision
2	Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation, and their potential application to decapod crustacean aquaculture (literature review)	**Jon I. L. Aquino**: Conceptualization, writing - original draft **Lisa Elliott**: Review & editing **Chaoshu Zeng**: Review & editing **Damien B.B.P. Paris**: Review & editing, supervision
3	Use of optimised conventional and advanced sperm quality diagnostic tools to establish reproductive data in wild-caught redclaw crayfish (*Cherax quadricarinatus*)	**Jon I. L. Aquino**: Conceptualisation, data curation, formal analysis, investigation, methodology, project administration, software, visualisation, writing - original draft, funding acquisition **Lisa Elliott**: Conceptualisation, funding acquisition, methodology, resources, supervision, writing - review & editing **Jodie Morris**: Methodology, visualisation, writing - review & editing **Rhondda Jones**: Data curation, formal analysis, writing - review & editing **Chaoshu Zeng**: Conceptualisation, funding acquisition, methodology, supervision **Damien B.B.P. Paris**: Conceptualisation, funding acquisition, methodology, supervision, writing - review & editing **John Cavalieri:** Conceptualisation, methodology, project administration, resources, visualisation, supervision, writing - review & editing

4	Quality of spermatozoa extracted from spermatophore removed by dissection or extruded by electroejaculation from wild-caught redclaw crayfish (*Cherax quadricarinatus*)	**Jon I. L. Aquino**: Conceptualisation, data curation, formal analysis, investigation, methodology, project administration, software, visualisation, writing - original draft, funding acquisition **Lisa Elliott**: Conceptualisation, funding acquisition, methodology, resources, supervision **Jodie Morris**: Methodology, visualisation, writing - review & editing **Rhondda Jones**: Data curation, formal analysis, writing - review & editing **Chaoshu Zeng**: Conceptualisation, funding acquisition, methodology, supervision **Leo Nankervis**: Review & editing **John Cavalieri:** Conceptualisation, methodology, project administration, resources, visualisation, supervision, writing - review & editing
5	Reproductive potential of male redclaw crayfish (*Cherax quadricarinatus*) fed with plant-based diets supplemented with astaxanthin and/or cholesterol	**Jon I. L. Aquino**: Conceptualisation, data curation, formal analysis, investigation, methodology, project administration, software, visualisation, writing - original draft, funding acquisition **Leo Nankervis**: Conceptualisation, resources, methodology, supervision, writing - review & editing **Jodie Morris**: Methodology, visualisation, writing - review & editing **Lisa Elliott**: Conceptualisation, funding acquisition, methodology, resources, supervision, writing - review & editing **Rhondda Jones**: Data curation, formal analysis **Chaoshu Zeng**: Conceptualisation, funding acquisition, methodology, supervision **John Cavalieri:** Conceptualisation, methodology, project administration, resources, visualisation, supervision, writing - review & editing
6	General discussion, conclusion, and future directions	**Jon I. L. Aquino**: Conceptualization, writing - original draft **John Cavalieri**: Review & editing, supervision

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Generative AI technology was not used in the preparation of any part of this thesis.

Data storage

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Table of contents

Acknowledgments iii

Publication records vi

Statement of the contribution of others viii

Statement of the Use of Generative AI xii

Data storage xiii

Table of contents xiv

List of Figures xx

List of Tables xxiii

List of Appendix xxv

List of Abbreviations xxvi

Abstract 1

Chapter 1. General introduction 5

1.1. Status of global aquaculture and crustacean production and consumption 5

1.2 Freshwater crayfish production 6

1.3. Redclaw aquaculture production 10

1.4. Present challenges in redclaw production 13

1.5. Role of sperm quality in reproduction and diagnostic tools 15

1.6. Research aims 17

1.7. References *20*

Chapter 2. Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation and their potential application to decapod crustacean aquaculture 25

2.1. Abstract *25*

2.2. Introduction *26*

2.3. Collection and evaluation of sperm quality in decapod crustaceans *30*

2.3.1. Spermatophore extraction and semen extenders 31

2.3.2. Advanced tools for sperm quality evaluation 46

2.3.2.a. Sperm number, morphology, and membrane integrity 46

2.3.2.b. Acrosome reaction 49

2.3.2.c. Mitochondrial function 52

2.3.2.d. Sperm DNA fragmentation 54

2.4. Sperm cryopreservation *61*

2.4.1. Cryoprotectants 71

2.4.2. Room temperature and chilled storage of crustacean spermatozoa 73

2.4.3. Long-term cryopreservation 74

2.4.4. Sperm vitrification 76

2.5. Natural and artificial fertilisation in decapod crustaceans *77*

2.5.1. Spermatophore structure and function 79

2.5.2. Natural fertilisation 80

2.5.3. Artificial fertilisation 86

2.6. Conclusions *88*

2.7. Acknowledgements *91*

2.8. References *91*

Chapter 3. Use of optimised conventional and advanced sperm quality diagnostic tools to establish reproductive data in wild-caught redclaw crayfish (*Cherax quadricarinatus*) 120

3.1. Abstract *120*

3.2. Introduction *121*

3.3. Material and Methods *124*

3.3.1. Experimental animals 124

3.3.2. Spermatophore extraction and sperm preparation 125

3.3.3. Sperm number, concentration, and morphology 126

3.3.4. Sperm viability assessment 128

3.3.5. DNA fragmentation assessment 132

3.3.6. Flow cytometry 135

3.3.7. Determination of total potential fertile sperm concentration (TPFSC) 136

3.3.8. Statistical analyses 136

3.4. Results *137*

3.4.1. Reproductive data of male fertility for redclaw crayfish 137

3.5. Discussion *145*

3.6. Conclusions *154*

3.7. Acknowledgements *154*

3.8. References *155*

Chapter 4. Quality of spermatozoa extracted from spermatophore removed by dissection or extruded by electroejaculation from wild-caught redclaw crayfish (*Cherax quadricarinatus*)....166

4.1. Abstract....166

4.2. Introduction....167

4.3. Material and Methods....171

4.3.1. Experimental animals....171

4.3.2. Experiment 1: Electroejaculation efficiency....171

4.3.3. Experiment 2: Sperm quality evaluation from spermatophores collected by electroejaculation and dissection....172

4.3.3.a. Sperm concentration....173

4.3.3.b. Sperm viability....173

4.3.3.c. DNA fragmentation assay....173

4.3.3.d. Flow cytometry....174

4.3.3.e. Determination of total potential fertile sperm concentration (TPFSC)....174

4.3.4. Statistical analysis....174

4.4. Results....175

4.4.1. Experiment 1: Electroejaculation efficiency....175

4.4.2. Comparing sperm quality between spermatophore collection methods....175

4.5. Discussion....178

4.6. Conclusions....185

4.7. Acknowledgements....185

4.8. References....186

Chapter 5. Reproductive potential of male redclaw crayfish (*Cherax quadricarinatus*) fed with plant-based diets supplemented with astaxanthin and/or cholesterol 194

5.1. Abstract 194

5.2. Introduction 195

5.3. Material and Methods 198

5.3.1. Animals 198

5.3.2. Feeding regime 199

5.3.2.a. Experimental design and diet preparation 199

5.3.2.b. Crayfish maintenance and sampling 201

5.3.2.c. Spermatophore extraction and sperm preparation 202

5.3.3. Sperm quality assessment 202

5.3.3.a. Sperm concentration 202

5.3.3.b. Sperm viability 202

5.3.3.c. DNA fragmentation assay 204

5.3.3.d. Flow cytometry 204

5.3.3.e. Calculation of total potential fertile sperm concentration (TPFSC), gonadosomatic (GSI) and hepatosomatic (HSI) indices 204

5.3.4. Statistical analyses 205

5.4. Results 206

5.5. Discussion 211

5.6. Conclusion 216

5.7. Acknowledgements 217

5.8. References 217

Chapter 6. General discussion, conclusions, and future directions......................... 227

6.1. General discussion 227

6.2 Future directions.. 230

6.3. Limitation of the study 231

6.4. Conclusions.. 232

6.5. References 233

Appendix 235

List of Figures

Figure 1.1. The natural distribution (shaded) of redclaw, *C. quadricarinatus* (Jones et al., 1996). 9

Figure 1.2. Traditional production cycle of redclaw, *C. quadricarinatus* (Jones, 2011)....11

Figure 2.1. Traditional and advanced predictive biomarkers of sperm quality and conditions that may contribute to improved male reproductive performance. 62

Figure 2.2. Electron (transmission and scanning) micrographs of the spermatophore wall and released spermatozoa in narrow-clawed crayfish (*A. leptodactylus*). (a) dissolving wall of the spermatophore with distorted granules (arrowheads) and areas where female glair secretions have penetrated (arrows); (b) exposed anterior acrosome secreting its contents in numerous droplets; (c) sagittal close-up showing acrosomal secretion with electron-dense vesicles at the inner surface of the acrosome being discharged extracellularly (d) a spermatozoon released from the extracellular capsule with nuclear radial arms and acrosomal complex clearly visible; (e) discharge of filaments from the anterior portion of the acrosome causing a cavity; (f) sagittal section of a spermatozoon extruding filaments and electron-dense material from the apical zone of the acrosome leaving a withdrawn subacrosomal zone; (g) close-up of electron-dense material being expelled via the apical zone; (h) cross-section of remaining acrosome layers and subacrosome zone after release of electron-dense materials; (i) boundary between subacrosome zone and nucleus (arrowheads). A, acrosome; Ac, acrosome complex; AM, acrosomal membrane; AZ, apical zone; EC, extracellular capsule; Fi, filaments; IL, inner acrosome layer; ML, middle acrosome layer; N, nucleus; PM, residual plasma membrane; RA, radial arms; SA, subacrosomal zone (Niksirat et. al., 2014).............85

Figure 2.3. Application of advanced reproductive tools to improve sustainable production of economically important decapod crustaceans in aquaculture.............90

Figure 3.1. Validation of nuclear staining specificity of (a, b) viability and (c, d) DNA fragmentation assays for *C. quadricarinatus* displaying intact/viable (blue; H_{342}^{+}), membrane-damaged/dead (pink; PI^{+}/ H_{342}^{+}), or DNA-damaged (green; $FITC^{+}$/ H_{342}^{+}) spermatozoa. FITC, fluorescein isothiocyanate; H_{342}, Hoechst 33342; PI, propidium iodide; *, acrosome; yellow arrows - nuclear region; scale bar = 50 µm. 130

Figure 3.2. Gating strategy for assessment of the viability of subpopulations of *C. quadricarinatus* spermatozoa by Hoechst 33342/propidium iodide staining and flow cytometry. (a) FSC-area and FSC-height were used to exclude debris and aggregates; (b) the sperm population was gated using an FSC-A and SSC-A contour plot. An H_{342}/PI scatter plot was then used to identify the following: (c) unlabeled sperm cells, U_1, (d) H_{342} single-stained positive control (H_{342}^{+}), (e) fixed and permeabilised PI single-stained positive control (PI^{+}), (f) fixed and permeabilised double-stained H_{342}/PI-positive control (H_{342}^{+}/PI^{+}), and (g) a representative test sample showing subpopulations of viable and dead spermatozoa. FSC, forward scatter; H_{342}, Hoechst 33342; PI, propidium iodide; SSC, side scatter. 132

Figure 3.3. Gating strategy for assessment of sperm DNA fragmentation of subpopulations of *C. quadricarinatus* spermatozoa by TUNEL and flow cytometry. (a) FSC-Area and FSC-Height were used to exclude debris and aggregates; (b) the sperm population was gated using an FSC-A and SSC-A contour plot. An H_{342}/TUNEL-FITC scatter plot was then used to identify the following: (c) unlabeled sperm cells, U_1; (d) unlabeled control with H_{342}, U_2; (e) negative control in label solution, N_1; (f) negative control in label solution with H_{342}, N_2; (g) *DNase*-treated FITC positive control, P_1; (h) *DNase*-treated FITC positive control with H_{342}, P_2; and (i) a representative test sample showing subpopulations of intact and DNA-damaged spermatozoa. FITC, fluorescein isothiocyanate; FSC, forward scatter; H_{342}, Hoechst 33342; SSC, side scatter..134

Figure 3.4. Sub-structure of the extruded spermatophore (SP) of *C. quadricarinatus*. (a) Intact spermatophore 10 min after extrusion (stereo microscopy); (b) H_{342}-stained spermatophore showing distinct primary and secondary layers 24 h after extrusion (inverted fluorescent microscopy); c) spermatophore showing distinct primary and secondary layers after 96 h (phase contrast); (d) spermatozoa liberated from a spermatophore after 96 h by gentle pipetting (phase-contrast). H_{342}, Hoechst 33342; PSL, primary spermatophore layer; SSL, secondary spermatophore layer; SP, spermatophore; SPZ, spermatozoa; scale bar = 50 μm (c and d). 138

Figure 3.5. Phase contrast micrographs of *C. quadricarinatus* spermatozoa. (a) Measurement of the sperm head (length and width) and presence of a putative tail-like structure (white arrows) (b) Hoechst 33342 stained nuclear area (yellow arrow) of spermatozoa and putative tail-like structure (white arrow). Scale bar = 50 μm. 139

Figure 3.6. Linear regressions with a 95% confidence interval (blue band) between (a) body weight and sperm viability, (b) spermatophore weight and sperm concentration, (c) spermatophore weight and total potential fertile sperm concentration, (d) spermatophore weight and sperm DNA fragmentation, and (e) sperm concentration and DNA fragmentation. All regressions are significant at $p \leq 0.05$. BW, body weight; SPW, spermatophore weight; TPFSC, total potential fertile sperm concentration.......... 140

Figure 4.1. The model prediction of spermatophore extrusion across body weights with a 95 % confidence interval (grey band) in wild-caught redclaw crayfish (*Cherax quadricarinatus*). The circles aligned with 1.00 signify redclaw males that produced spermatophores, while those aligned with 0.00 did not. The blue line indicates the proportion of spermatophore production based on the body weight. Logistic regression is significant at $p \leq 0.05$. p, probability of extracting spermatophore.......... 177

Figure 5.1. The schematic diagram of the experimental design aimed to evaluate supplementation with a plant-based diet with either astaxanthin, cholesterol, or both ingredients on the reproductive potential of male redclaw broodstock during a supplementation period of 60 days. AX - astaxanthin; CHO - cholesterol. 203

List of Tables

Table 1.1. Annual production and value of major freshwater crustaceans produced by aquaculture from 2010-2017 (FAO-FIGIS, 2019). 8

Table 1.2. Redclaw-producing countries by earnings with countries producing low to zero (shaded) redclaw over time (expressed per thousand US$; FAO-FIGIS, 2019). 13

Table 2.1. Methods of spermatophore extraction, handling, semen extenders and sperm quality evaluation reported for decapod crustaceans. 33

Table 2.2. Room temperature and chilled storage, long-term cryopreservation and vitrification of spermatophore and spermatozoa in decapod crustaceans. 63

Table 3.1. Characterisation of sperm morphology in wild-caught adult redclaw crayfish (*C. quadricarinatus*). 142

Table 3.2. Reproductive values of sperm quality for wild-caught adult redclaw crayfish (*C. quadricarinatus*). 143

Table 3.3. Relationship between sperm quality parameters and body and spermatophore weights in wild-caught adult redclaw crayfish (*C. quadricarinatus*). 144

Table 4.1. Comparison of sperm quality (mean ± SEM) from spermatophores collected by dissection and electroejaculation from wild-caught redclaw crayfish using a paired T-test analysis at $p \leq 0.05$ statistical significance (*Cherax quadricarinatus*). 176

Table 5.1. Composition of the experimental diet. 200

Table 5.2. Reproductive performance of male redclaw broodstock (*C. quadricarinatus*) subjected to four different dietary treatments at three different time points. Data are presented as mean ± SEM. 208

Table 5.3. Sperm quality indices and other reproductive potential parameters for all dietary treatments combined over the duration of feeding in male redclaw (*C. quadricarinatus*) broodstock. Data are presented as mean ± SEM..209

Table 5.4. Change in reproductive potential parameters over feeding duration after acclimation, including all dietary treatments combined in male redclaw (*C. quadricarinatus*) broodstock. Data are presented as mean ± SEM..210

List of Appendix

Appendix A. Statistical summary on the effect of an un-supplemented control diet and dietary supplementation of astaxanthin (AX), cholesterol (CHO) or both AX and CHO on reproductive potential in male redclaw (*C. quadricarinatus*) broodstock between treatments at Days 0, 30, and 60. This appendix also includes the changes in reproductive potential parameters over feeding duration after acclimation for all dietary treatments combined. 235

List of Abbreviations

AC	Alternating current
ACH	Australian Crayfish Hatchery
AITHM	Australian Institute of Tropical Health and Medicine
AX	Astaxanthin
BW	Body weight
CHO	Cholesterol
Ca^{2+}	Calcium
CFS	Calcium-free saline
CL	Carapace length
cm	Centimetres
CPHMVS	College of Public Health, Medical and Veterinary Sciences
CRC	Cooperative Research Centre
CRCNA	CRC for Developing Northern Australia
CW	Carapace width
°C	Degrees Celsius
DF	DNA fragmented spermatozoa
DNA	Deoxyribonucleic acid
DVD	Distal vas deferens
EDTA	Ethylenediaminetetraacetic acid
Ex	Excitation
Em	Emission
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GSI	Gonadosomatic index
GW	Gonad's weight
h	Hour
HSI	Hepatosomatic index
HW	Hepatopancreas weight
H_3BO_3	Boric acid
H_{342}	Hoechst 33342
JCUPRS	James Cook University Postgraduate Research Scholarship
KCl	Potassium chloride
L	Longest distance of an ellipse
MARFU	Marine and Aquaculture Research Facility
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
mL	Millilitre
NaCL	Sodium chloride
NaOH	Sodium hydroxide
n	Number of

NO_2^--N	Nitrite
NO_3^--N	Nitrate
N_1/N_2	Negative control stain
PI	Propidium iodide
P_1/P_2	Positive control stain
ppm	Parts per million
ppt	Parts per thousand
r	Pearson's correlation coefficient
ROS	Reactive oxygen species
RT	Room temperature
SC	Sperm concentration
SD	Standard deviation
SEM	Standard error of the mean
SP	Spermatophore weight
SSC	Side scatter
TAN; NH_4^+-N/ NH_3-N	Total ammonia nitrogen
TL	Total body length
TPFSC	Total potential fertile sperm concentration
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
U_1/U_2	Unstained control
µl	Microliter
VD	Vas deferens
VS	Viable spermatozoa
W	Widest distance perpendicular to sperm head length

Abstract

Increasing global human population and seafood consumption, coupled with fully exploited fisheries, have led to a growing dependence on aquaculture. As a result, aquaculture has become the fastest-growing food-producing sector. Redclaw crayfish (*Cherax quadricarinatus*) has emerged as a commercially attractive option for aquaculture production due to its fast growth, large size, robustness, and high yield of saleable meat from individuals. However, traditional earthen pond breeding has provided an inconsistent supply of early juveniles due to low survival and limited production. An intensive breeding facility has been established, but its juvenile output is unpredictable. To date, the influence of broodstock fertility on juvenile production has never been investigated, and there are few reports on the characteristics of sperm quality in redclaw. This thesis investigated the mechanisms of quantifying and optimising sperm quality for redclaw broodstock by characterising reproductive data in wild-caught crayfish, comparing sperm quality from spermatophores collected using two practical methods, and assessing sperm quality in male redclaw fed with a broodstock diet supplemented with astaxanthin and cholesterol.

To explore the role of sperm quality in redclaw fertility, existing knowledge and reproductive technologies to evaluate potential male fertility in other decapod crustaceans were reviewed (detailed in Chapter 2). Traditional measures of male reproductive performance, such as sperm concentration and morphology, have limitations in evaluating large numbers of spermatozoa and males. Analysis of sperm quality in crustaceans can be improved by optimising diagnostic sperm biomarkers commonly used in mammals, which may provide a better understanding of overall reproductive performance. Diagnostic tools to evaluate sperm quality involve fluorescent nuclear dyes and high-throughput flow cytometry to measure

sperm viability, mitochondrial function, acrosome reaction, and DNA fragmentation. This review offers detailed insights into the current advances in evaluating sperm quality, sperm cryopreservation, and artificial fertilisation techniques, with potential applications in crustacean farming. Based on this review, optimised diagnostic tools were selected to assess sperm quality in redclaw crayfish.

In Chapter 3, optimised sperm diagnostic tools were developed to establish reproductive data in wild-caught redclaw crayfish. Sexually mature male redclaw (n = 33) were collected from the wild and subjected to electroejaculation to produce spermatophores to evaluate sperm quality. Results showed that the mean ± SEM sperm viability, DNA fragmentation, concentration, and total potential fertile sperm concentration (TPFSC) were 65.2 ± 3.9 %, 17.2 ± 2.5 %, 42.5 x 10^4 ± 5.1 x 10^4 cells/mL, and 23.6 x 10^4 ± 3.4 x 10^4 cells/mL, respectively. In addition, the first comprehensive description of sperm morphometry and a presumptive, tail-like structure was reported in redclaw crayfish. Spermatophore weight was positively correlated with sperm concentration ($r = 0.58; p = 0.002$) and TPFSC ($r = 0.57; p = 0.0004$) but not with sperm viability ($p = 0.188$) and was inversely correlated with sperm DNA fragmentation ($r = 0.42; p = 0.0218$). Sperm viability ($r = 0.46; p = 0.010$) was negatively correlated with body weight. This study reported the first quantitative reproductive traits on sperm morphometry and function, validated species-specific diagnostic tools for sperm quality and provided insights into the relationship between body and spermatophore weights and sperm quality for animals largely collected from a single site at a specific time of the year. This information may help screen highly fertile male breeders and enhance strategies for harvesting spermatozoa for selective breeding programs in commercial redclaw aquaculture.

In Chapter 4, the efficiency of collecting spermatophores using a single electroejaculation protocol at different body weights was investigated. The sperm quality from spermatophores collected using this method was compared with those isolated by dissection from redclaw crayfish using the conventional and advanced sperm quality diagnostic tools developed in Chapter 3. In Experiment 1, 153 sexually mature redclaw males (> 40.0 g) were subjected to electroejaculation to assess efficiency in extruding spermatophores. In experiment 2, 33 male crayfish weighing 99.4 ± 7.6 g (mean ± SEM) underwent assessment of sperm quality assessment after isolation of spermatophores by dissection and electroejaculation. The odds of extracting spermatophore (p) were affected by body weights ($p = 0.029$) and were greatest within crayfish weighing between 60 and 130 g. The success rate in extracting spermatophores from either gonopore using electroejaculation was 22.2 % (n = 153). The mean weight of spermatophores from the left (0.016 ± 0.002 g) and right (0.011 ± 0.002 g) gonopores did not differ ($p = 0.430$) and was not significantly influenced by body weights ($p = 0.272$). A greater mean concentration of spermatozoa, sperm viability, TPFSC and lower sperm DNA fragmentation was measured in spermatozoa extracted from spermatophores after dissection compared to those extracted after electroejaculation ($p < 0.001$). These findings indicate that the method of spermatophore collection affected sperm quality in redclaw crayfish. Spermatophores isolated by dissection offer better sperm quality, making them preferable for developing advanced breeding techniques, including sperm cryopreservation and artificial fertilisation. Electroejaculation is, however, a non-lethal option for collecting spermatophores, which could be used for regular breeding and commercial production of redclaw in the future.

Studies investigating the dietary requirement for redclaw broodstock are limited and focused only on juveniles, while dietary effects on sperm quality in redclaw have not been

undertaken. Therefore, the influence of the inclusion of the antioxidant astaxanthin (AX) and/or the steroid cholesterol (CHO), which have both been reported to influence reproductive function in female redclaw, in a plant-based diet on sperm quality was investigated in a 60-day feeding trial (Chapter 5). Included in the study were four experimental diets (n = 48/treatment) formulated and prepared to contain either no additional AX or CHO or formulated to contain either 100 mg/kg AX, 10 g/kg CHO, or both 100 mg/kg AX and 10 g/kg CHO. Reproductive performance indicators, including sperm viability, concentration, spermatophore weight, GSI, and HSI, all increased over the duration of the experiment ($p < 0.05$). In contrast, sperm DNA fragmentation doubled over the same period ($p < 0.001$). Overall, implementation of a plant-based broodstock diet supplemented with AX or CHO accelerated gonadal maturation within 30 days, indicating earlier attainment of reproductive capacity, albeit with decreased sperm DNA integrity after 60 days. Notably, the broodstock basal diet formulation in this study may prove beneficial for future breeding technologies to enhance gamete quality in intensive hatchery systems.

This thesis presented the first optimised method to comprehensively assess sperm quality in redclaw crayfish, establishing reproductive data, including sperm morphometry and quality. Results further demonstrated that methods used to collect spermatophores can impact sperm quality, which could affect breeding management strategies, especially when utilising male gametes for sperm banking and artificial reproduction. In this thesis, we demonstrated that dietary supplementation with AX or CHO improved gonadal maturity to reproductive conditions within a 30-day window, but unidentified factors increased sperm DNA fragmentation after 60 days, which is a concern and requires further investigation. Additionally, these studies highlighted potential areas for future research to improve the management of male broodstock in the commercial production of redclaw crayfish.

Chapter 1. General introduction

1.1. Status of global aquaculture and crustacean production and consumption

The global demand for seafood is increasing due to population growth, and the consumption per capita has increased from 9.9 kg in 1960 to 20.2 kg in 2020 (WHO, 2019; FAO, 2022). The global fisheries and aquaculture production, excluding aquatic plants, reached an all-time high in 2020, with 49 % coming from aquaculture (FAO, 2022). From 1990 to 2020, the FAO (2022) reported a 60.6 % increase in seafood production, with global production reaching about 177.8 million tonnes in 2020, excluding aquatic plants. The economic value of this production was worth over US$ 406 billion in that year, of which US$ 265 billion was derived from aquaculture production. As set by the 2030 Agenda for Sustainable Development of the United Nations, the production of world fisheries and aquaculture is projected to expand continuously, with global aquatic animal production exceeding 202 million tonnes, to supply seafood for an average per capita consumption of 21.4 kg by 2030 (FAO, 2022). This demand cannot be met solely by wild fisheries, which have plateaued in volume since the mid-1990s. As a result, an expanding aquaculture industry has emerged to bridge the shortfall. Aquaculture has surpassed wild fisheries production since 2013 and is predicted to supply more than 50 % of the world's seafood by 2030 (FAO, 2022). Hence, aquaculture has a crucial role in supporting the seafood industry's growth (FAO, 2022).

Global aquaculture production was dominated by farmed finfish in 2020, reaching a volume of 57.5 million tonnes (with a total value of US$146.1 billion). Molluscs came in second with a volume of 17.7 million tonnes (US$ 29.8 billion), followed by crustaceans with a volume of 11.2 million tonnes (US$ 81.5 billion; FAO, 2022). Though crustaceans ranked third in

production by volume, their production earned a higher gross farm gate value than molluscs and generated high-value export products similar to finfish. There is a growing interest in farming freshwater crustaceans, with production increasing continuously since 2000 (FAO, 2022). In 2020, freshwater crustacean aquaculture accounted for almost 35 % of total crustacean production, with the main freshwater crustaceans produced being crayfish, prawns, and crabs (FAO, 2022).

1.2 Freshwater crayfish production

In 2017, crayfish were the most farmed and profitable group of freshwater crustaceans, contributing 1.2 million tonnes (US$ 10 billion), an increase of more than 200 % from 2010 (Table 1.1; FAO-FIGIS, 2019). The increasing popularity of crayfish is due to their affordable price and desirable taste and texture, which is highly comparable to other, more expensive marine crustaceans (Wang et al., 2015). They have many of the same characteristics as marine crustaceans, but they can be farmed in a broader range of geographies and with less expensive production methods than marine crustaceans (Wei et al., 2023). While they are more affordable than marine crustaceans, they nonetheless provide a high-value crop attractive to farmers (FAO, 2022). In China, freshwater crayfish aquaculture is a multi-billion-dollar industry that provides a livelihood for millions of people (Li, 2018; Liqiang and Kun, 2018). Once regarded as pests by rice farmers, freshwater crayfish are now one of the country's most sought-after crustacean commodities and culinary delicacies (Ge, 2018; Wei et al., 2023). Normal farming practices in China involve extensive rice and crayfish rotation followed by more intensive crayfish farming after the rice is harvested. This enables efficient utilisation of space needed for agriculture and aquaculture production simultaneously (Wang et al., 2015; Wei et al., 2023). Given the rising price and demand in

local and international markets, freshwater crayfish farming in China is considered a lucrative industry (Wang et al., 2015; Ge, 2018; Li, 2018; Liqiang and Kun, 2018). In 2020, the dominant freshwater crayfish produced was the red swamp crawfish (*Procambarus clarkii*; FAO, 2022), but these are relatively small and yield low meat recovery (15 %; McClain et al., 2007).

Redclaw crayfish are an excellent choice for commercial aquaculture, making it an alternative species for aquaculture to the red swamp crawfish, among other freshwater crayfish. It has become increasingly popular among aquaculture producers (Medley et al., 1994; Masser and Rouse, 1997; Jones, 1998; Núñez-Amao et al., 2018). It is a large and robust freshwater crustacean species with a wide geographic distribution in northern Australia and southern Papua New Guinea (Figure 1.1). It has a simple life cycle and requires relatively modest production technology. It is a non-burrowing species that grows quickly, can tolerate a wide range of environmental conditions, and is less aggressive as an adult form than other *Cherax* and decapod species (Medley et al., 1994; Jones, 1998, 2011). It has an average meat recovery of 28 – 30 % of its body weight, which is relatively higher than that of red swamp crawfish and other farmed *Cherax* species (Bitomsky, 2008). This gives consumers the choice of a more affordable option that tastes similar to other expensive crustaceans in the global seafood market (Medley et al., 1994; Jones et al., 1996; Masser and Rouse, 1997; Jones, 1998, 2011; Ghanawi and Saoud, 2012).

Table 1.1. Annual production and value of major freshwater crustaceans produced by aquaculture from 2010 – 2017 (FAO-FIGIS, 2019).

Freshwater species	Year															
	2010		2011		2012		2013		2014		2015		2016		2017	
	Kilo tonnes	US$ billion	Kilo tonnes	US$ billion	Kilo tonnes	US$ billion	Kilo tonnes	US$ billion	Kilo tonnes	US$ billion	Kilo tonnes	US$ billion	Kilo tonnes	US$ billion	Kilo tonnes	US$ billion
Crayfish	596.9	3.4	513.6	3.4	549.6	4.0	597.7	4.7	659.6	5.4	720.5	5.8	895.2	7.4	1,193.8	10.0
Crab	572.4	5.0	613.6	6.3	650.7	7.2	663.6	8.0	722.7	9.2	747.4	9.4	748.8	9.5	751.0	9.5
Prawns	465.2	2.8	452.4	3.2	470.3	3.5	527.4	4.3	457.9	3.9	466.5	3.9	530.3	4.3	581.4	4.7

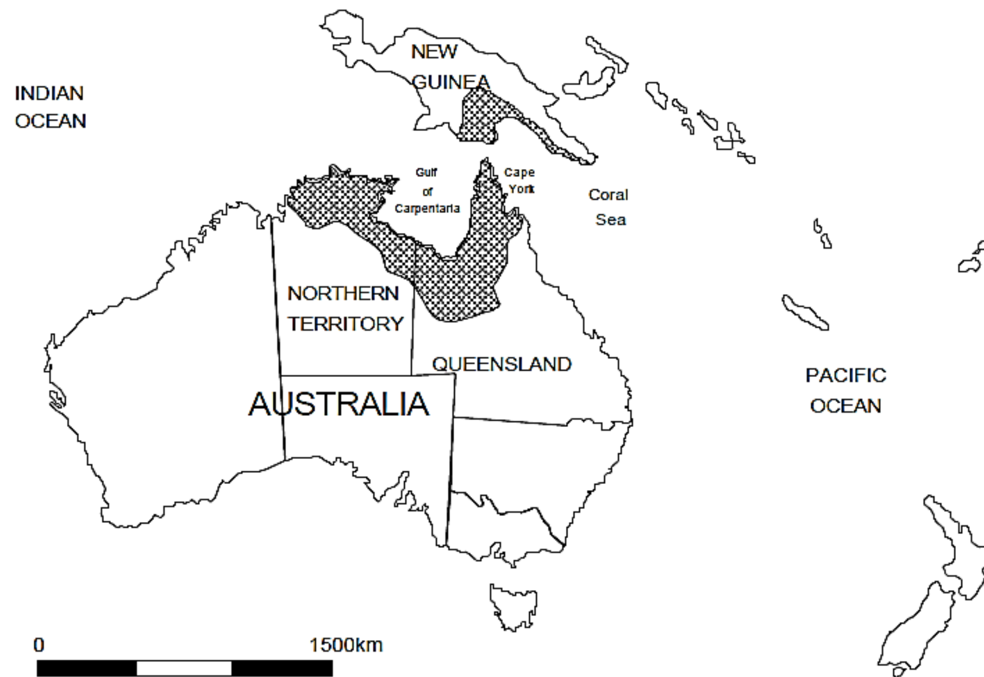


Figure 1.1. The natural distribution (shaded) of redclaw, *C. quadricarinatus* (Jones et al., 1996).

In warmer regions of Australia, redclaw crayfish is one of the farmed species, together with freshwater fish, barramundi, and marine prawns (Schofield and Lewis, 2022). The history of redclaw production started in 1975 at a small scale until its potential as an aquaculture commodity was more intensively explored in the late 1980s (Jones, 1990; Jones et al., 1996). Between 2021 and 2022, there were 19 farms producing redclaw of around 31.5 tonnes (US$ 543,000; Schofield and Lewis, 2022). Apart from Australia, redclaw is also farmed in Southeast Asia, Central and South America and the USA. Most products are currently marketed locally in each region but exhibit high export potential (Jones, 2011; Irvin et al., 2018; Mobsby, 2018).

1.3. Redclaw aquaculture production

Traditionally, the entire production cycle of redclaw has been limited to pond aquaculture. The production cycle involves two phases: 1) the production of early juveniles and 2) the growing of juveniles to marketable size (Figure 1.2). In Phase 1, redclaw are reared directly in earthen ponds for juvenile production without an indoor hatchery phase (Jones et al., 1996; Jones, 2011). Juvenile production starts by stocking ponds with adult and mature broodstock at a ratio of 1 male to 4 females and at a stocking density of 0.2/m^2 or egg-bearing (“berried”) females (Jones et al., 1996; Jones and Valverde, 2020). Under favourable conditions, one hectare can produce 60,000 – 120,000 advanced juveniles at approximately 50 – 100 per adult female after 3 – 4 months (Jones et al., 1996; Jones, 2000). The most critical factors determining survival are shelter and food since early juveniles are highly cannibalistic. Redclaw juveniles ranging from 5 – 15 g are harvested (Phase 2), sorted into similar sizes and stocked in grow-out ponds for rearing for the next generation of breeders and for the market

(Jones et al., 1996; Masser and Rouse, 1997; Jones, 1998; Jones and Ruscoe, 2000; Jones, 2011).

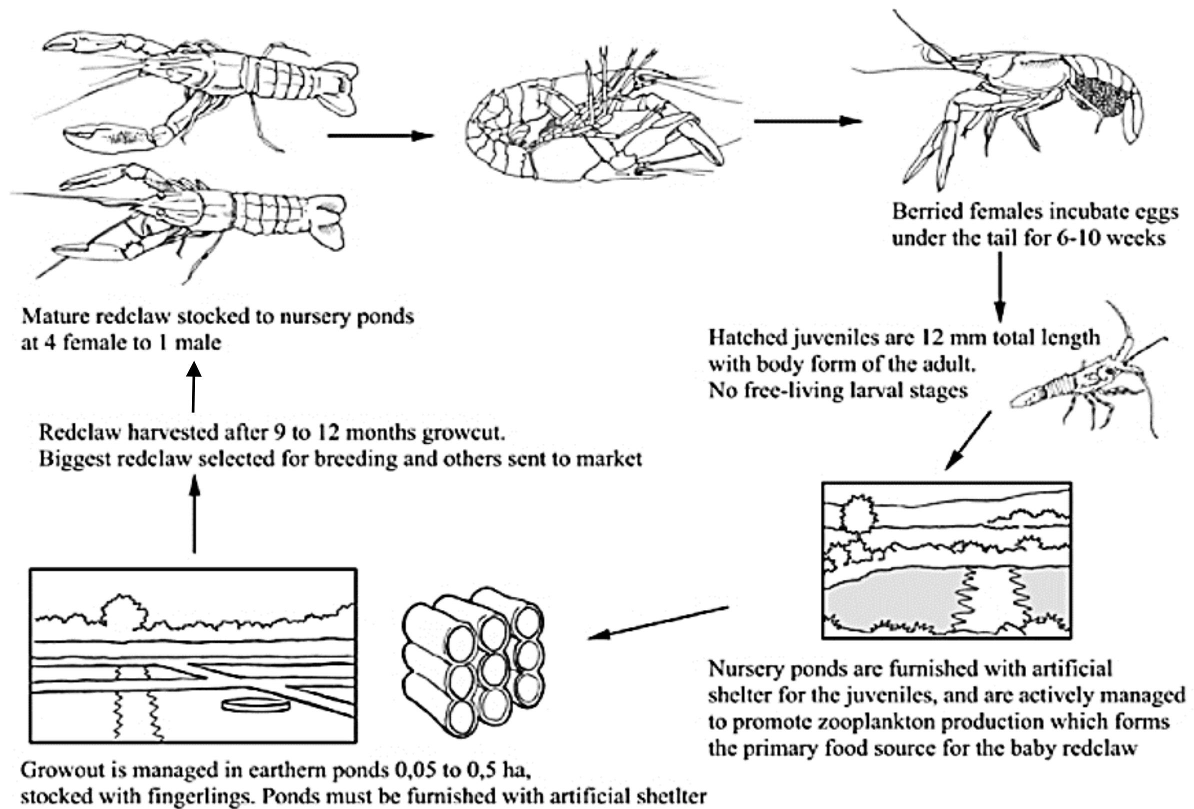


Figure 1.2. Traditional production cycle of redclaw, *C. quadricarinatus* (Jones, 2011).

The grow-out of redclaw farming adopts a semi-intensive approach. It typically utilises rectangular earthen ponds constructed in compact clay soils ranging from 0.1 – 0.5 ha with a depth of 1 – 2.5 m (Medley et al., 1994; Jones et al., 1996; Jones, 2011). Ponds are designed to be maintained with high water levels but have a secondary canal in the middle to allow rapid and complete water drainage. Net fencing is installed around the pond to avoid redclaw escaping and entry of predators (e.g., herons and cormorants; Jones et al., 1996). Some ponds are lined with coarse river gravel to provide a base where crevices are formed between stones, serving as additional shelters for redclaw. Artificial shelters such as synthetic mesh,

net bundles and stacks of pipe are often provided as shelters at 0.3/m^2 (Jones, 2000; Jones and Ruscoe, 2001). An airlift pump is typically installed for water aeration and operates at night. However, other equipment, such as paddle wheels, can also be used, but at a higher cost. A holding tank must be included in the setup to hold harvested stock prior to market or for further pond culture (Jones et al., 1996; Jones, 1998; Jones and Ruscoe, 2001; Jones, 2011).

Redclaw crayfish are eurythermal (21 – 32 ºC), mesohaline (0 – 12 ppt) and can tolerate low dissolved oxygen (DO) levels down to 1 ppm. They survive at pH 6.5 – 9.0, total ammonia and nitrite concentration of < 25 and < 10 ppm, respectively, water hardness > 40 ppm, and iron and manganese content < 0.1 ppm (King, 1994; Jones, 1995b; Meade and Watts, 1995; Jones et al., 1996; Masser and Rouse, 1997; Jones, 2000, 2011; Núñez-Amao et al., 2018). However, they become susceptible to diseases when culture conditions are sub-optimal. To date, major disease outbreaks are rare in Australia. Nevertheless, biosecurity measures such as quarantine of newly acquired broodstock, maintenance of optimal water parameters, and good health management to prevent the outbreak of disease are still important and need to be implemented (Medley et al., 1994; Jones et al., 1996; Jones, 1998; Longshaw, 2011).

Indoor systems for the grow-out of redclaw have also been trialled but are generally not considered economically viable given that most food items for redclaw come from decaying matter and plankton that naturally populate earthen ponds (Jones, 2011). Thus, earthen ponds for grow-out operations involve lower investment and operational costs. To date, no reports have documented the successful commercial production of redclaw in a tank-based system; however, further research is needed on the economic viability of alternative culture systems (Jones, 2011).

1.4. Present challenges in redclaw production

Led by Australia and Malaysia in 2017, eight countries have produced redclaw for the market since 2007 (Table 1.2). Despite the potential for profit, the redclaw farming industry still faces multiple challenges, including insufficient juvenile supply, a limited production season, and a generally small production scale (Jones, 2011; FAO-FIGIS, 2019). Earnings have also declined in several countries although the reasons for this have not been reported (Table 1.2).

Table 1.2. Redclaw-producing countries by earnings with countries producing low to zero (shaded) redclaw over time (expressed per thousand US$; FAO-FIGIS, 2019).

Country	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Australia	1,216	938	887	877	937	820	712	614	784	997	1,303
Malaysia	0	0	0	0	0	0	615	540	886	1 219	1,097
Mexico	162	103	32	12	59	2,714	37	103	159	136	0
New Caledonia	0	66	63	60	63	58	60	60	50	0	0
Ecuador	300	300	3,000	0	0	0	0	0	0	0	0
Argentina	161	138	7	0	0	0	0	0	0	0	0
Uruguay	19	29	2	0	2	2	2	0	0	0	0
Barbados	6	6	6	6	6	6	6	6	6	6	6
Grand total	1,865	1,580	3,998	954	1,066	3,600	1,432	1,323	1,885	2,359	2,406

One of the leading causes of the inconsistent and low production of redclaw is the limited supply of juvenile seedstock for large-scale farming, thereby restricting the profitability of each pond from its full potential. The traditional redclaw hatchery-nursery system typically yields low early juvenile survival (5 – 10 %) and is limited mainly to the warm season (5 – 6 months each year; Jones et al., 1996; Jones, 2011), making production during the winter months problematic (Jones, 1995a; Masser and Rouse, 1997; Jones, 2011; Irvin et al., 2018).

The existing breeding and juvenile production system does not allow farmers to closely monitor and manage the age and development of each batch of juveniles, which results in variability in crayfish size in earthen ponds. This problem causes significant losses due to high cannibalism, in which smaller and newly moulted crayfish are often predated upon (Jones, 1995a; Parnes and Sagi, 2002).

To mitigate these challenges, an intensive breeding facility for juvenile production was established (Jones and Valverde, 2020). Eggs are obtained from berried females in earthen ponds or, more recently, breeder tanks and stocked in an incubator until they hatch. However, despite these efforts, inconsistent production still exists due to high variability in the survival rates of redclaw juveniles (Jones and Valverde, 2020; Rigg et al., 2020).

Methods for early juvenile production and grow-out have been established, but farming technology needs further refinement to facilitate the expansion of the redclaw crayfish industry. The absence of large numbers of intensively produced early juveniles for stocking grow-out ponds exacerbates the production shortage. The high level of cannibalism and variable growth rates among juveniles limit the capacity of the stakeholders to realise the potential of redclaw aquaculture. In addition, numerous studies have been conducted on the dietary requirements of juvenile redclaw, but none of them has been implemented on a commercial scale and requires further optimisation (Rigg et al., 2020 and references therein). Alongside these challenges, inbreeding has been observed to occur in redclaw aquaculture (Stevenson et al., 2013). Increasing evidence suggests that broodstock fertility affects embryonic development and larval output in finfish (Bobe and Labbé, 2010; Migaud et al., 2013). This could also be the case for redclaw broodstock, where underlying sub-fertility problems may contribute to fluctuations in productivity.

1.5. Role of sperm quality in reproduction and diagnostic tools

Male fertility can significantly influence the breeding success of crustaceans in captivity (Chamberlain and Lawrence, 1981; Leung-Trujillo and Lawrence, 1987). One method to assess the fertility potential of male broodstock is to quantify sperm quality (Harlıoğlu et al., 2018a). Spermatozoa quality can be retrospectively determined by their capacity to yield viable embryos when in contact with good-quality eggs under favourable environmental conditions (Bobe and Labbé, 2010). Other aquaculture species use fertilisation rates to represent male reproductive potential, but this technique has not been established for redclaw crayfish. While estimating fertility rates is effective, a major disadvantage is that data is retrospectively captured, which delays the screening of males for breeding programs. In addition, several variables could potentially influence the results, including the inherent fertility of the exposed females, egg quality, spermatozoa/egg ratio and incubation time and other environmental and nutritional conditions which add complexity, time and expense in designing standardised and robust methodology (Cabrita et al., 2009). Sperm quality is a critical but often overlooked factor contributing to crustacean hatchery success. The energetic investment in broodstock is primarily through female egg development, while spermatozoa are viewed as a simple DNA delivery mechanism. However, it is becoming increasingly evident that poor sperm quality, specifically due to DNA damage, can impede fertilisation and lead to poor reproductive outcomes (Pérez-Cerezales et al., 2010; Simon et al., 2014; Duangjai et al., 2023). As a result, commercial aquaculture has been considering the assessment of sperm quality as an early diagnostic tool to assess the potential fertility of male breeders for selective breeding programs, which could help reduce the cost of production (Cabrita et al., 2009; Feng, 2018).

Spermatozoa from crustaceans are morphologically diverse, have no midpiece, and are non-flagellated (Lewis and Ford, 2012; Harlıoğlu et al., 2018a). Hence, while sperm motility is used as an indicator of sperm viability in finfishes and other vertebrates, it cannot be applied to decapod crustaceans such as redclaw crayfish (Lezcano et al., 2004; Aquino et al., 2022). In addition, the absence of a standard size and morphological appearance of spermatozoa in decapod crustaceans decreases the ability to objectively differentiate the quality of spermatozoa using optical microscopy (Lezcano et al., 2004). These characteristics of redclaw spermatozoa necessitate the use of more objective, reliable and advanced alternative methods to acquire results faster and with greater accuracy and consistency (Cabrita et al., 2014).

Alternative methods that can be applied to the evaluation of sperm quality in crustaceans include the assessment of sperm concentration, viability, and DNA integrity, which were presented in details in Chapter 3. For instance, sperm viability can be determined by assessing the integrity of the plasma membrane using fluorescent nuclear membrane-staining dyes, coupled with flow cytometry to rapidly analyse > 10,000 spermatozoa within a single sample using more biological samples (Cabrita et al., 2009; Cabrita et al., 2014). DNA fragmentation assessment using a similar approach has also been used to identify subfertile spermatozoa with otherwise normal morphology that can lead to unhatched eggs or impaired larval development (Peña et al., 2017; Castelo-Branco et al., 2018; Duangjai et al., 2023). Recently, efforts have been made to improve the sensitivity of these approaches to estimate potential fertilising capacity in decapod crustacean aquaculture, specifically, in this thesis, for redclaw spermatozoa (Feng et al., 2018; Feng et al., 2019; Duangjai et al., 2023).

1.6. Research aims

The review and studies presented in this thesis investigate the mechanisms of quantifying and optimising sperm quality for redclaw crayfish broodstock. In order to do this, the series of studies reported in this thesis were conducted to answer the following key research questions:

1) What is the quality of spermatozoa from male redclaw crayfish caught in the wild?

2) Can electroejaculation provide an alternative and less invasive method of collecting spermatophores from male redclaw breeders than dissection without compromising sperm quality?

3) Can a plant-based diet supplemented with astaxanthin (AX), cholesterol (CHO), or both improve the reproductive potential of male redclaw crayfish?

The hypotheses tested to fill knowledge gaps and address the challenges in assessing sperm quality in male redclaw crayfish were as follows:

1) Sperm quality tools could be modified and developed to quantify sperm quality in redclaw male broodstock.

2) The odds of collecting spermatophores from male redclaw crayfish using electroejaculation is similar for crayfish weighing > 40 g. In addition, there is no difference in sperm quality from spermatophores removed by dissection or extruded by electroejaculation.

3) Feeding male redclaw broodstock with diets supplemented with AX, CHO, or both nutrients could enhance their reproductive potential, including sperm quality, spermatophore weight and gonadosomatic and hepatosomatic indices.

This thesis is outlined as follows:

Chapter 1: General Introduction

This chapter provides an overview of the industry, research questions, tested hypotheses and thesis outline.

Chapter 2: Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation, and their potential application to decapod crustacean aquaculture.

This chapter reviews existing knowledge and reproductive technologies to assess potential male fertility in decapod crustaceans. This review identifies diagnostic tools to assess sperm quality in redclaw crayfish.

Chapter 3: Use of optimised conventional and advanced sperm quality diagnostic tools to establish reproductive data in wild-caught redclaw crayfish (*Cherax quadricarinatus*).

This chapter presents developed and optimised redclaw-specific diagnostic tools for establishing reproductive data on sperm quality in wild-caught redclaw male broodstock.

Chapter 4: The quality of spermatozoa extracted from spermatophore removed by dissection or extruded by electroejaculation from wild-caught redclaw crayfish (*Cherax quadricarinatus*)

To further assess the quality of redclaw spermatozoa, a protocol for collecting spermatophores from male redclaw broodstock is investigated in this chapter. The efficiency of collecting spermatophores using electroejaculation is tested. Sperm quality from spermatophores collected following electroejaculation with those isolated by dissection from redclaw crayfish is compared using conventional and advanced sperm quality diagnostic tools.

Chapter 5: The reproductive potential of male redclaw crayfish (*Cherax quadricarinatus*) fed with plant-based diets supplemented with astaxanthin and/or cholesterol

Low reproductive potential in males sourced from the wild based on sperm quality was detected after establishing the reproductive data in redclaw (Chapter 3). Hence, in this chapter we report on the evaluation of the effects of dietary supplementation with AX and/or CHO and the duration of dietary treatments on reproductive performance indicators, including sperm quality, spermatophore weight, and gonadosomatic and hepatosomatic indices in redclaw male broodstock over 60 days.

Chapter 6: General Discussion, Conclusions and Future directions

This chapter discusses the overall finding of this thesis and sets future directions for the advance of breeding management for redclaw aquaculture.

1.7. References

Bitomsky, J., 2008. Scoping report- Redclaw industry development, in: Lofthouse, L. (Ed.). 36 Grafton Street, Cairns, QLD, 4870, pp. 22-40.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165(3), 535-548. https://doi.org/https://doi.org/10.1016/j.ygcen.2009.02.011.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V., 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432, 389-401. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.04.034.

Cabrita, E., Robles, V., Herráez, M.P., 2009. Sperm quality assessment, in: Cabrita, E., Robles, V., Herráez, M.P. (Eds.), Methods in Reproductive Aquaculture: Marine andFreshwater Species. Biology Series. CRC Press (Taylor and Francis Group), Boca Raton, Florida, USA, pp. 93-148.

Castelo-Branco, T., Guerra, M.M.P., Soares, R., Peixoto, S., 2018. Sperm vitrification of *Litopenaeus vannamei*: effect of cryoprotectant solutions on sperm viability and spawning quality after artificial insemination. Aquac. Int. 26(3), 913-920. https://doi.org/https://doi.org/10.1007/s10499-018-0258-y.

Chamberlain, G., Lawrence, A.L., 1981. Maturation, reproduction, and growth of *Penaeus vannamei* and *P. stylirostris* fed natural diets. J. World Maric. Soc. 12, 207-224.

Duangjai, E., Umyoo, P., Meunpol, O., Puanglarp, N., 2023. Sperm quality assessment of white shrimp (*Litopenaeus vannamei*) broodstock using comet assay. Trends Sci. 20(6), 4768. https://doi.org/10.48048/tis.2023.4768.

FAO-FIGIS, 2019. Global fisheries and aquaculture production 1950 - 2017, Fisheries Global Information System (FIGIS). FI Institutional Websites. Statistics and Information Branch. Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153, Rome.

FAO, 2022. The state of world fisheries and aquaculture 2022. Towards blue transformation, The State of World Fisheries and Aquaculture (SOFIA). FAO, Rome, Italy, p. 266.

Feng, T., 2018. Spermatogenesis and Sperm Assessment in the Black Tiger Prawn, *Penaeus monodon*, School of Agriculture and Food Sciences. University of Queensland, Queensland, Australia, p. 195.

Feng, T., Gosálvez, J., Lopez-Fernandez, C., Arroyo, F., Paterson, B., Johnston, S., 2018. Sperm chromatin dispersion test (SCDt) for the assessment of sperm DNA

fragmentation in black tiger prawn, *Penaeus monodon*. Aquaculture 491, : 281-288. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.03.041.

Feng, T., Paterson, B., Coman, G., Johnston, S., 2019. Evaluating the impact of moulting and chilled storage of spermatophores on the integrity of plasma membrane, acrosome and DNA of black tiger prawn (*Penaeus monodon*) spermatozoa. Aquac. Res. 50(1), 226-235. https://doi.org/https://doi.org/10.1111/are.13888.

Ge, C., 2018. China's craving for crayfish creates US$2 billion business, South China Morning Post South China Morning Post China.

Ghanawi, J., Saoud, I.P., 2012. Molting, reproductive biology, and hatchery management of redclaw crayfish *Cherax quadricarinatus* (von Martens 1868). Aquaculture 358-359, 183-195. https://doi.org/https://doi.org/10.1016/j.aquaculture.2012.06.019.

Harlıoğlu, M.M., Farhadi, A., Gür, S., 2018. Determination of sperm quality in decapod crustaceans. Aquaculture 490, : 185-193. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.02.031.

Irvin, S., Coman, G., Musson, D., Doshi, A., Stokes, C., 2018. Aquaculture viability. A technical report to the Australian Government from the CSIRO Northern Australia Water Resource Assessment, part of the National Water Infrastructure Development Fund: Water Resource Assessments, CSIRO, Australia.

Jones, C.M., 1990. The biology and aquaculture potential of the tropical freshwater crayfish, *Cherax quadricarinatus*. Department of Primary Industries Queensland, Brisbane, QLD, Australia.

Jones, C.M., 1995a. Production of juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae) I. Development of hatchery and nursery procedures. Aquaculture 138(1), 221-238. https://doi.org/https://doi.org/10.1016/0044-8486(95)00068-2.

Jones, C.M., 1995b. Salinity tolerance of the tropical freshwater crayfish, C*herax quadricarinatus* (von Martens)(Decapoda, Parastacidae), Freshwater Crayfish VIII: Papers from the 8th International Symposium on Astacology. 8th International Symposium on Astacology, Baton Rouge, LA, USA, pp. 399-409.

Jones, C.M., 1998. Redclaw crayfish, in: Hyde, K.W. (Ed.), The New Rural Industries: A Handbook for Farmers and Investors. Rural Industries Research and Development Corporation, Canberra, ACT, Australia, pp. 127-133.

Jones, C.M., 2000. Recommended practices for redclaw crayfish aquaculture based on research and development activities, 1988 through 2000. Northern Fisheries Centre, Department of Primary Industries and Fisheries, Cairns QLD 4870, Australia.

Jones, C.M., 2011. Cultured aquatic species information programme- *Cherax quadricarinatus*, Cultured Aquatic Species Information Programme, 13 January 2011 ed. FAO Fisheries and Aquaculture Department, Rome.

Jones, C.M., Ruscoe, I.M., 2000. Assessment of stocking size and density in the production of redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda: Parastacidae), cultured under earthen pond conditions. Aquaculture 189(1), 63-71. https://doi.org/https://doi.org/10.1016/S0044-8486(00)00359-8.

Jones, C.M., Ruscoe, I.M., 2001. Assessment of five shelter types in the production of redclaw crayfish *Cherax quadricarinatus* (Decapoda: Parastacidae) under earthen pond conditions. J. World Aquac. Soc. 32(1), 41-52. https://doi.org/10.1111/j.1749-7345.2001.tb00920.x.

Jones, C.M., Ruscoe, I.M., Queensland, F.F.a.A.C., 1996. Production technology for redclaw crayfish (*Cherax quadricarinatus*) : Final report. Freshwater Fisheries and Aquaculture Centre, Department of Primary Industries, Walkamin, QLD.

Jones, C.M., Valverde, C., 2020. Development of mass production hatchery technology for the redclaw crayfish, *Cherax quadricarinatus*. Freshw. Crayfish 25(1), 1 - 6. https://doi.org/10.5869/fc.2020.v25-1.001.

King, C.R., 1994. Growth and survival of redclaw crayfish hatchlings *(Cherax quadricarinatus* von Martens) in relation to temperature, with comments on the relative suitability of *Cherax quadricarinatus* and Cherax destructor for culture in Queensland. Aquaculture 122(1), 75-80. https://doi.org/https://doi.org/10.1016/0044-8486(94)90335-2.

Leung-Trujillo, J.R., Lawrence, A.L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65(3), 363-370. https://doi.org/https://doi.org/10.1016/0044-8486(87)90249-3.

Lewis, C., Ford, A.T., 2012. Infertility in male aquatic invertebrates: A review. Aquat. Toxicol. 120-121, : 79-89. https://doi.org/https://doi.org/10.1016/j.aquatox.2012.05.002.

Lezcano, M., Granja, C., Salazar, M., 2004. The use of flow cytometry in the evaluation of cell viability of cryopreserved sperm of the marine shrimp (*Litopenaeus vannamei*). Cryobiology 48(3), 349-356. https://doi.org/https://doi.org/10.1016/j.cryobiol.2004.03.003.

Li, W., 2018. Crayfish was China's most popular dish in 2017, GB Times. GB Times, China.

Liqiang, H., Kun, L., 2018. Demand for crawfish knows no bounds, Chinadaily. Chinadaily, China.

Longshaw, M., 2011. Diseases of crayfish: A review. J. Invertebr. Pathol. 106(1), 54-70. https://doi.org/https://doi.org/10.1016/j.jip.2010.09.013.

Masser, M.P., Rouse, D.B., 1997. Australian red claw crayfish, Southern Regional Aquaculture Center Publication. United States Department of Agriculture, Cooperative States Research, Education, and Extension Service, United States of America, pp. 1-7.

McClain, W.R., Romaire, R.P., Lutz, C.G., Shirley, M.G., 2007. Louisiana crawfish production manual. Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Louisiana Cooperative Extension Service, p. 57.

Meade, M.E., Watts, S.A., 1995. Toxicity of ammonia, nitrite, and nitrate to juvenile Australian crayfish, *Cherax quadricarinatus*. J. Shellfish Res. 14(2), 341-346.

Medley, P.B.C., Jones, C.M., W. Jr Avault, J., 1994. A global perspective of the culture of Australian redclaw crayfish, *Cherax quadricarinatus*: Production, economics and marketing. J. World Aquac. Soc. 25(4), 6-13.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Rev. Aquac. 5(s1), S194-S223. https://doi.org/https://doi.org/10.1111/raq.12025.

Mobsby, D., 2018. Australian fisheries and aquaculture statistics 2017, Fisheries Research and Development Corporation Project 2018-134. ABARES, Canberra, December. CC BY 4.0, pp. 1-94.

Núñez-Amao, L., Naranjo-Páramo, J., Hernández-Llamas, A., Vargas-Mendieta, M., Villarreal, H., 2018. Estimating production costs of preadult redclaw crayfish*, Cherax quadricarinatus*, reared in a commercial nursery system: A stochastic bioeconomic approach. J. World Aquac. Soc. 50(1), 172-185. https://doi.org/10.1111/jwas.12554.

Parnes, S., Sagi, A., 2002. Intensification of redclaw crayfish *Cherax quadricarinatus* culture. I. Hatchery and nursery system. Aquac. Eng. 26(4), 251-262. https://doi.org/https://doi.org/10.1016/S0144-8609(02)00034-1.

Peña, S.T., Jr., Gummow, B., Parker, A.J., Paris, D.B.B.P., 2017. Revisiting summer infertility in the pig: could heat stress-induced sperm DNA damage negatively affect early embryo development? Anim. Prod. Sci. 57(10), 1975-1983. https://doi.org/https://doi.org/10.1071/AN16079.

Pérez-Cerezales, S., Martínez-Páramo, S., Beirão, J., Herráez, M.P., 2010. Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 139(6), 989. https://doi.org/10.1530/rep-10-0037.

Rigg, D., Seymour, J., Courtney, R., Jones, C., 2020. A review of juvenile redclaw crayfish *Cherax quadricarinatus* (von Martens, 1898) production in aquaculture. Freshw. Crayfish 25(1), 13-30. https://doi.org/10.5869/fc.2020.v25-1.013.

Schofield, R., Lewis, S., 2022. Ross Lobegeiger report to farmers: aquaculture production summary for Queensland 2021 - 2022. Department of Agriculture and Fisheries, Fisheries Queensland, Department of Agriculture and Fisheries, pp. 1-12.

Simon, L., Murphy, K., Shamsi, M.B., Liu, L., Emery, B., Aston, K.I., Hotaling, J., Carrell, D.T., 2014. Paternal influence of sperm DNA integrity on early embryonic development. Hum. Reprod. 29(11), 2402-2412. https://doi.org/https://doi.org/10.1093/humrep/deu228.

Stevenson, J., Jerry, D., Owens, L., 2013. Redclaw selective breeding project, Rural Industries Research and Development Corporation. Rural Industries Research and Development Corporation, Rural Industries Research and Development Corporation Level 2, 15 National Circuit BARTON ACT 2600 p. 58.

Transparency-Market-Research, 2018. Crayfish market- global industry analysis, size, share, growth, trends, and forecast 2016 - 2024, 7 June 2019 ed. Transparency Market Research, State Tower, 90 State Street, Suite 700, Albany NY - 12207, United States.

Wang, Q., Cheng, L., Liu, J., Li, Z., Xie, S., De Silva, S.S., 2015. Freshwater aquaculture in PR China: trends and prospects. Rev. Aquac. 7(4), 283-302. https://doi.org/10.1111/raq.12086.

Wei, Y., Müller, D., Sun, Z., Lu, M., Tang, H., Wu, W., 2023. Exploring the emergence and changing dynamics of a new integrated rice-crawfish farming system in China. Environ. Res. Lett. 18(6), 064040. https://doi.org/10.1088/1748-9326/acd8d2.

WHO, 2019. Global and regional food consumption patterns and trends, in: Organization, W.H. (Ed.) World Health Organization. Department of Nutrition for Health and Development (NHD), World Health Organization, Avenue Appia 20, 1211 Geneva 27, Switzerland.

Chapter 2. Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation and their potential application to decapod crustacean aquaculture

2.1. Abstract

To maximise productivity, a better understanding of the underlying causes of subfertility that lead to inferior offspring and high mortality is imperative. In decapod crustaceans, most research has focused on female reproductive performance, with little attention given to male fertility. Paternal genetic contribution is critical to both successful embryonic and post-embryonic development. Assessment of sperm quality can be a direct method to determine male subfertility in decapods. Sperm quality parameters such as sperm concentration and morphology have traditionally been used to determine male reproductive performance, but these procedures are time-consuming and can only assess a limited number of sperm cells and males. Alternative diagnostic biomarkers used widely in humans and other mammals could be adapted to decapod crustaceans and may be more indicative of sperm fertilisation competence and male reproductive performance. These predictive biomarkers use fluorescent cellular dyes and high-throughput flow cytometry or computer-assisted sperm microscopic analysis to evaluate sperm viability, mitochondrial function, acrosome reaction, and DNA fragmentation. This review examines current and advanced biomarkers to evaluate sperm quality and further explores state-of-the-art procedures of sperm cryopreservation (conventional *vs*. vitrification techniques) and artificial fertilisation in decapod crustaceans. Sperm freezing coupled with artificial fertilisation in decapods permits the long-term storage, controlled timing and selection of individuals for reproduction. Collectively, these tools can

be applied to commercial broodstock management to improve productivity and accelerate selective breeding in the crustacean aquaculture industry.

Keywords: Artificial fertilisation, decapod crustaceans, male fertility tools, sperm cryopreservation, sperm quality, subfertility

2.2. Introduction

More than 50% of the world's seafood products presently come from aquaculture (FAO, 2020); with crustaceans making up the bulk of seafood produced that typically command high values in the global market (FAO-FIGIS, 2019; FAO, 2020). Production of crustaceans through aquaculture has been increasing over the past decades (FAO, 2020) and by 2018, total global aquaculture production reached 9.4 million tonnes and was valued at US$ 69.3 billion.

Reproductive efficiency in crustacean aquaculture is generally focused on the performance of female broodstock, with little attention given to male fertility (Harlıoğlu and Farhadi, 2017). However, several reports have shown that sperm quality, as well as spermatophore formation, could affect reproductive efficiency in decapod crustaceans (Sandifer and Lynn, 1980; Chow, 1982; Sandifer et al., 1984; Chow et al., 1985; Bart et al., 2006; Vuthiphandchai et al., 2007; Morales-Ueno et al., 2013). Hence, screening male broodstock for fertility is critical to achieving high fertilisation rates and yielding good-quality offspring. Such screening can detect the presence of sub- or infertile male broodstock in the hatchery, which may reduce production efficiency and raise operational costs (Feng, 2018).

A direct way to determine male fertility is to evaluate sperm quality (Feng, 2018; Marc et al., 2021). Sperm quality refers to the capacity of spermatozoa to fertilise eggs successfully and yield normal embryos (Bobe and Labbé, 2010; Harlıoğlu et al., 2018). Evaluation of sperm quality in crustaceans to date is limited to conventional methods that include light microscopy for sperm concentration and morphometry, as well as measurement of gonad and spermatophore weights (Bugnot and López Greco, 2009a, 2009b; Harlioğlu et al., 2012; Harlioğlu et al., 2013; Farhadi et al., 2018; Farhadi, A. and Harlıoğlu, M.M., 2019; Farhadi et al., 2019a). Predictive biomarkers developed for assessing sperm quality in vertebrates, such as plasma membrane integrity, acrosome reaction, mitochondrial function, and DNA fragmentation, can be modified and applied to invertebrates (Lewis and Ford, 2012). Meanwhile, traditional parameters, such as sperm concentration and morphometry, can be analysed using advanced diagnostic tools, including Computer-Assisted Sperm Analysis (CASA) and/or flow cytometry, which can produce highly accurate and fast results. (Lezcano et al., 2004; Bobe and Labbé, 2010; Lewis and Ford, 2012; Figueroa et al., 2016; Harlıoğlu et al., 2018).

In parallel with the development of advanced assessment tools for sperm quality, the freezing of gametes, particularly spermatozoa, is a significant step in advancing the aquaculture industry (Cloud and Patton, 2009; Cabrita et al., 2014). Sperm cryopreservation technology paves the way for controlling the timing of reproduction and enables the genetic diversity of broodstock to be maintained in frozen banks. Conservation of genetics from wild stocks is a critical component of breeding programs in aquaculture, preventing potential loss of genetic diversity (and hence fitness) through the under-representation of founders over multiple generations of captive breeding (Cloud and Patton, 2009). Moreover, cryopreservation of spermatozoa in liquid nitrogen (LN_2) for extended periods of time guarantees a year-round

supply of high-quality spermatozoa with minimal effort and space, thereby reducing hatchery costs required for male broodstock maintenance, as well as facilitating selective breeding (Gwo, 2000; Cabrita et al., 2014).

In crustacean aquaculture, animals often only reach sexual maturity and reproduce in captivity when conditions are favourable. Moreover, captive males and females of some species do not mature and reproduce synchronously (Mylonas et al., 2017; Beirão et al., 2019). With the intensification of aquaculture, interest in controlling crustacean reproduction using assisted breeding techniques, such as artificial fertilisation, has gained impetus (Cabrita et al., 2014; Beirão et al., 2019). Artificial fertilisation involves the manual collection and handling of spermatozoa (in a way that maintains their quality) in order to fertilise eggs *in vivo* or *in vitro* with maximum efficiency (Beirão et al., 2019). Gametes can be collected from superior broodstock with high-quality phenotypic traits and used in artificial fertilisation during a precisely controlled time window compared to natural mating, which could take several hours to days (Beirão et al., 2019). By so doing, the genetic selection for high-quality offspring can also be maximised, especially in commercial production.

In reproduction, the contribution of intact sperm DNA to the embryo is critical to ensure the healthy development of offspring (Fernández-Díez et al., 2016; Fernández-Díez and Herráez, 2018; Erraud et al., 2019). Until recently, male fertility has been assessed microscopically using traditional sperm quality parameters, such as sperm morphology and sperm concentration (using a counting chamber). However, these methods can be time-consuming and can only evaluate a limited number of sperm cells and males (Lezcano et al., 2004). Even spermatozoa in high concentrations that look morphologically normal according to traditional assessment methods may not be capable of fertilising eggs (Ahmadi and Ng, 1999; Devaux et

al., 2011; Castelo-Branco et al., 2018; Fernández-Díez and Herráez, 2018; Erraud et al., 2019). As such, successful reproduction also entails high fertilisation competence, which can be more precisely evaluated through the assessment of intracellular organelle functions, such as sperm plasma membrane integrity, mitochondrial function, acrosome reaction, and DNA fragmentation.

Given that the fertility of male decapod crustaceans has received little attention, to date, no comprehensive review currently exists that specifically focuses on the evaluation, cryopreservation and artificial fertilisation of spermatozoa for these highly important aquacultural species. A recent review by Beirão et al. (2019) included a broad range of aquatic species, such as finfish, bivalve molluscs, and marine mammals, and only penaeid shrimp represented decapod crustaceans (Beirão et al., 2019). Moreover, that review only focussed on current techniques for sperm collection, storage, and artificial insemination (Beirão et al., 2019). This review hence attempts to fill the gap by focusing on recent advances for evaluating sperm quality to determine male fertility that could potentially be applied to decapod crustaceans, including those advanced techniques developed for mammals, as well as the current state of sperm cryopreservation and artificial fertilisation techniques and their implications for crustacean aquaculture. The development and optimisation of such fertility and assisted reproductive techniques may improve productivity in the crustacean aquaculture industry by early diagnosis of infertility and acceleration of selective breeding.

2.3. Collection and evaluation of sperm quality in decapod crustaceans

In general, the male reproductive system of crustaceans consists of testes and vas deferens (VD) that connect to external openings called gonopores, elevated genital papillae in some species or an extruding copulatory structure in others (Wilson, 2009; Subramoniam, 2017a). Spermatogenesis in the decapod testis begins with the proliferation of spermatogonia and subsequent meiosis, yielding primary and secondary spermatocytes that eventually differentiate into spermatids. Through spermiogenesis, spermatids develop into mature spermatozoa that are then transported to the vas deferens, where they are gradually coated with 1 – 3 spermatophore layers during transit (An et al., 2011; Subramoniam, 2017a). The spermatophore itself is a complex structure comprised of sperm-filled tubes coated with layers of a protective gelatinous matrix (Subramoniam, 2017c). During copulation, spermatophores are extruded by the male through the paired gonopores located at the base of the walking legs and deposited inside the sex organ or attached to the ventral surface of the female, which may store it for a prolonged period prior to fertilisation (Subramoniam, 2017c; Beirão et al., 2019).

As such, in order to obtain spermatozoa to evaluate their quality, extraction of a spermatophore from the male reproductive tract is necessary (Beirão et al., 2019). Most knowledge in this regard is derived from economically important decapod crustaceans targeted for aquaculture. Not surprisingly, however, current sperm handling protocols vary greatly from one species to another (Beirão et al., 2019).

2.3.1. Spermatophore extraction and semen extenders

Commonly, spermatophore extraction involves anaesthetising adult males at 10 °C for 15 – 20 min and weighing them before using one of the following three methods to collect spermatophores. The first and most commonly adopted method involves post-mortem dissection of the reproductive tract, particularly the vas deferens, to expose the spermatophore; the size, colour and consistency of which are examined macroscopically. For freshwater crayfish, often at least a 1 cm section of the distal vas deferens (DVD) is cut, and spermatophore is squeezed gently using a fine tweezer into a tube containing 1 ml of physiological saline solution specially formulated for freshwater crustaceans (Bugnot and López Greco, 2009a, 2009b). This size of DVD is similar to the spermatophore being transmitted to the female naturally during copulation in freshwater crayfish (Bugnot and López Greco, 2009a, 2009b; Harlioğlu et al., 2012; Harlioğlu et al., 2013). The two other methods of spermatophore collection, i.e., manual extrusion and electroejaculation, are non-lethal and hence have the benefits of avoiding killing valuable male broodstock (Beirão et al., 2019; Diggles, 2019). Manual extrusion, commonly used in sexually mature male penaeids, can be performed by applying gentle pressure with the thumb and index finger laterally around the coxas of the fifth pair of walking legs of the male (Beirão et al., 2019; Diggles, 2019). Frequent manual extrusion may cause inflammation of the genitals and deposition of melanin (melanisation); triggered by the presence of haemocytes embedded around the connective tissue of the genitals (Braga et al., 2018). Moreover, the reproductive tract can be damaged by melanisation of gonopores and reduced sperm quality if manual extrusion is performed incorrectly or too frequently (Braga et al., 2018).

Electroejaculation can be an alternative method to stimulate the extrusion of spermatophores from the gonopores of mature male decapods, including freshwater crayfish, freshwater prawns, and lobsters. Electroejaculation is done by placing two electrodes at the base of the sternal keel near the coxa of the fifth walking leg of the male (Aiken et al., 1984; Harris and Sandifer, 1986; Jerry, 2001). In the freshwater crayfish, *Cherax destructor*, an AC variable transformer delivered a maximum 55-V stimulus through a pair of electrodes at a maximum of 10 s pulses between 40 – 60 Hz to the male to induce muscle contractions, which led to the discharge of spermatophores (Jerry, 2001; Diggles, 2019). While manual extrusion may be a more straightforward procedure, its success depends on the experience and skill of the handler as well as the species. For example, manual extrusion is generally ineffective on hard-shelled crustaceans, such as freshwater crayfish and marine lobsters, for which electroejaculation may be the only non-lethal alternative (Kooda-Cisco and Talbot, 1983; Aiken et al., 1984; Sandifer et al., 1984; Jerry, 2001). By contrast, the intensity of electrical stimulation can be precisely controlled for electroejaculation, often leading to more consistent results (Kooda-Cisco and Talbot, 1983; Aiken et al., 1984; Sandifer et al., 1984; Jerry, 2001). The procedure of electroejaculation is believed to cause relatively limited discomfort to animals as long as low currents are used. Moreover, it is also considered safe and repeatable, provided that animals are handled carefully and allowed sufficient time to recover between extractions (Harris and Sandifer, 1986; Braga et al., 2018). Further methods include manual removal of spermatophores from the spermatheca, genitals or sternum of copulated female crustaceans using tweezers (Chow, 1982; Pongtippatee et al., 2007; Sarker et al., 2009; Zhang et al., 2010; Xu et al., 2014) and Table 2.1 summarises these various methods of spermatophore extraction reported in the literature.

Table 2.1. Methods of spermatophore extraction, handling, semen extenders and sperm quality evaluation reported for decapod crustaceans.

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
			Freshwater crab			
Somanniathelphusa pax.; Geothelphusa dehaani	Post-mortem dissection	Testis	Fixed in Davidson's solution	Gonadosomatic index (GSI)	Tissue weight	(Yamaguchi et al., 2008)
Eriocheir sinensis	Post-mortem dissection	Spermatophore	Precooled (4 °C) Ca^{2+}-free artificial seawater	Cryopreservation- induced acrosome reaction (AR)	Light microscopy	(Kang et al., 2009)
Eriocheir sinensis	Post-mortem dissection	Spermatophore, sperm	Ca^{2+} - filtered artificial seawater (FASW)	Acrosin activity, spermatophore digestion using accessory sex gland proteins	Scanning & transmission electron microscopy (SEM & TEM)	(Hou et al., 2010)
Eriocheir sinensis	Post-mortem dissection	Sperm	Ca^{2+} - FASW	AR	*In vitro* AR assay	(Li et al., 2010)
Sinopotamon henanense	Post-mortem dissection	Testis	1:9 (w/v) 0.86% saline solution at 4 °C.	Oxidative stress & apoptotic changes in testes against cadmium toxicity	Haematoxylin & eosin (H&E) staining, acridine orange (AO) / ethidium bromide (EB) dual fluorescent staining, TEM & DNA fragmentation analysis	(Wang et al., 2011)
Sinopotamon henanense	Post-mortem dissection	Seminal vesicle	Phosphate buffer saline (PBS; pH= 7.4)	Oxidative damage & ultrastructural changes after cadmium exposure	TEM; Malondialdehyde, MDA), proteins (protein carbonyl derivates, PCO) & DNA (DNA–protein crosslinks, DPC) biomarkers for oxidative damage	(Ma et al., 2013)
Barytelphusa Guerini	Post-mortem dissection	Testis	Ice-cold PBS	Morphology & GSI	Tissue weight; light microscopy	(Prasad et al., 2014)
Sinopotamon henanense	Post-mortem dissection	Sperm	PBS	Sperm count, oxidative damage, sperm plasma-membrane, AR, DNA integrity	Flow cytometry, fluorescent microscopy, reactive oxygen species (ROS), total antioxidant capacity (T-AOC), lipid peroxidation (MDA)	(Li et al., 2016)
			Freshwater crayfish			

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Cambaroides japonicas	Post-mortem dissection	Testis	5 °C in 1% OsO_4 (pH 7.2), veronal acetate for EM	Nuclear & cytoplasmic differentiation in sperm (sperm morphology)	TEM	(Yasuzumi et al., 1961)
Cambaroides japonicus	Post-mortem dissection	Testis	4% buffered formaldehyde (pH 7.4), 1% OsO_4 (pH 7.4) for EM	Microtubular structure & sites of thiamine pyrophosphatase activity in premature sperm (sperm morphology)	Light microscopy & TEM	(Yasuzumi and Lee, 1966)
Cambarus sp.	Post-mortem dissection	Testis, vas deferens	Sodium eaeodylate buffered 5% glutaraldehyde (pH 7.3), 2% OsO_4 for EM	Acrosome formation, transformation of mitochondria & development of microtubules in sperm (sperm morphology)	TEM	(Anderson and Ellis, 1967)
Astacus astacus	Post-mortem dissection	Vas deferens	Fixed in 2.5% glutaraldehyde in Sorensen's buffer solution (pH 7.2)	Sperm morphology	TEM	(López-Camps et al., 1981)
Cherax Tenuimanus; Cherax albidus	Post-mortem dissection; Electroejaculation	Sperm	Not mentioned	Sperm morphology	Light microscopy & TEM	(Beach and Talbot, 1987)
Cherax albidus	Post-mortem dissection	Vas deferens	Fixed in Bouin's solution	Spermatophore formation	Light microscopy & TEM	(Talbot and Beach, 1989)
Orconectes propincus; Janus frontalis	Post-mortem dissection	Sperm	0.5% glutaraldehyde & 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.6) for EM; Hoechst 33258 for DNA localisation	Microfilament DNA localisation	Fluorescent microscopy; Laser scanning confocal microscopy (LSCM); SEM	(Dupre and Schatten, 1993)
Procambus paeninsulanus	Post-mortem dissection	Testis	Paraformaldehyde-glutaraldehyde fixative in 0.1 M phosphate buffer pH 7.2	Sperm morphology & spermatogenesis	Light microscopy & TEM	(Hinsch, 1993)
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens	Fixed in Bouin's solution	Male reproductive tract morphology	Tissue weight & light microscopy	(Khalaila et al., 1999)
Cherax destructor	Electroejaculation	Spermatophore	Not mentioned	Spermatophore morphology, ejaculation efficiency	No. of males extruding spermatophores from both gonopores	(Jerry, 2001)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Cherax quadricarinatus	Post-mortem dissection	Androgen gland (AG), testis, vas deferens	Fixed in Bouin's solution	AG, testis & vas deferens weights, AG polypeptides	Tissue weight; histology; SDS-PAGE	(Khalaila et al., 2002)
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens spermatophore	Fixed in Bouin's solution	Male reproductive tract morphology	Histology	(López Greco et al., 2007)
Cherax quadricarinatus	Post-mortem dissection	Spermatophore	Fixed in Bouin's solution	Structural changes in spermatophore	Histology	(López Greco and Lo Nostro, 2008)
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens, spermatophore, sperm	Physiological salt solution; Fixed in Bouin's solution	Sperm count & mortality, reproductive tract weight & morphology	Tissue weight; vital dye exclusion (10% methylene blue); histology	(Bugnot and López Greco, 2009a)
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens spermatophore, sperm	Physiological salt solution; Fixed in Bouin's solution	Sperm count & mortality, reproductive tract weight & morphology	Vital dye exclusion (10% methylene blue); histology	(Bugnot and López Greco, 2009b)
Astacus leptodactylus	Post-mortem dissection	Testis, vas deferens	Fixed in Bouin's solution	Morphology	Light microscopy	(Erkan et al., 2009)
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens	Fixed in Bouin's solution	GSI, male reproductive tract morphology	Tissue weight & histology	(Vazquez and López-Greco, 2010)
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens	Fixed in Bouin's & Carnoy's solutions	Male reproductive tract morphology	Feulgen staining; light microscopy; TEM	(An et al., 2011)
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens	Not mentioned	Male reproductive tract morphology	Light microscopy	(Tropea et al., 2011)
Cherax quadricarinatus	Post-mortem dissection	Testis	Liquid nitrogen	Heat shock protein 70 influence on spermatogenesis	Real-time quantitative PCR	(Fang et al., 2012)
Austropotamobius italicus	Post-mortem dissection	Spermatophore	0.9% physiological saline	Sperm viability & longevity	Phase-contrast microscopy; Fluorescence microscopy	(Galeotti et al., 2012)
Astacus leptodactylus	Post-mortem dissection	Whole male reproductive tract	Physiological solution for freshwater crustaceans	Sperm number, male reproductive tract weight, testis & vas deferens weights, GSI, testicular index.	Tissue weight & light microscopy	(Harlioğlu et al., 2012)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Astacus leptodactylus	Post-mortem dissection	Spermatophore, sperm	Not mentioned	Sperm number, reproductive tract morphology, GSI & testicular index (TI)	Spermatophore weight & light microscopy	(Harlioğlu et al., 2013)
Cherax quadricarinatus	Post-mortem dissection	Testis	Liquid nitrogen (LN_2)	Prohibitin characterization	Gene expression; western blot analysis; immunofluorescent microscopy;	(Fang et al., 2013)
Orconectes sp.; Procambarus sp.; Astacus sp.	Post-mortem dissection; Electroejaculation	Sperm	2.5% glutaraldehyde in 0.1M phosphate buffer	Sperm ultrastructure	TEM	(Niksirat et al., 2013)
Astacus leptodactylus	Electroejaculation	Spermatophore	Not mentioned	Post-mating spermatophore morphology	SEM & TEM	(Niksirat et al., 2014a)
Pacifastacus leniusculus	Electroejaculation	Spermatophore	LN_2	Proteomic profiling	Two dimensional electrophoresis & Western blotting	(Niksirat et al., 2014b)
Cherax quadricarinatus; Cherax destructor	Electroejaculation	Spermatophore	2.5% glutaraldehyde in 0.1 M phosphate buffer	Comparative sperm ultrastructure	TEM	(Kouba et al., 2015)
Astacus astacus	Electroejaculation	Spermatophore	LN_2	Protein profiling	In-gel trypsin digestion & high-resolution mass spectrometry	(Niksirat et al., 2015b)
Pacifastacus leniusculus	Electroejaculation	Spermatophore	LN_2	Post-mating protein profile & pattern of protein tyrosine phosphorylation	Two-dimensional electrophoresis & Western blotting	(Niksirat et al., 2016)
Cambarus robustus; Orconectes propinquus; Orconectes rusticus	Post-mortem dissection	Sperm	2.5% glutaraldehyde in 0.1M phosphate buffer	Sperm ultrastructure	TEM	(Yazicioglu et al., 2016)
Procambarus clarkii	Post-mortem dissection	Testis	Not mentioned	Kinesin-14 motor protein KIFC1function during spermatogenesis	Rapid-Amplification of cDNA Ends (RACE), *In situ* hybridisation	(Ma et al., 2017)
Pontastacus leptodactylus	Post-mortem dissection	Whole reproductive tract, vas deferens, testis	0.9% NaCl solution	Sperm count, GSI, testicular index, vas deferens index	Tissue weight & light microscopy	(Farhadi and Harlıoglu, 2018)
Pontastacus leptodactylus	Post-mortem dissection	Distal ductus deferens	0.9% NaCl solution	Sperm count, incubation time & temperature for extraction	Light microscopy	(Farhadi et al., 2018)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Pontastacus leptodactylus	Post-mortem dissection	Vas deferens, testis	Ca^{2+}- free saline	GSI, vas deferens index, testicular index	Tissue weights; haemocytometer counts; light microscopy	(Farhadi, A. and Harlıoğlu, M.M., 2019)
Pontastacus leptodactylus	Electroejaculation	Spermatophore	Tris buffer solution	Artificial extrusion, artificial insemination, sperm count	Eosin–nigrosin staining; light microscopy	(Farhadi et al., 2019b)
Pontastacus leptodactylus	Post-mortem dissection	Whole reproductive tract	Not mentioned	Reproductive tract weight, testis weight, vas deferens weight, GSI, testicular index, vas deferens index, sperm count	Eosin–nigrosin staining; light microscopy; tissue weights	(Farhadi et al., 2019a)
Freshwater prawn						
Macrobrachium rosenbergii	Electroejaculation with 24h interval, gentle pressure on terminal ampullae post-mortem	Spermatophore	Buffered seawater (pH 7.4)	Artificial fertilisation, fertilisation rate	Fluorescent microscopy & SEM	(Sandifer and Lynn, 1980)
Macrobrachium rosenbergii	Manual removal from female's sternum	Spermatophore	Ringer solution at 2 °C or room temperature	Artificial insemination & short – term preservation	Fertilisation & hatching rates	(Chow, 1982)
Macrobrachium rosenbergii	Manual removal from female's sternum	Spermatophore	10% glycerol in freshwater or physiological saline (pH 7.6)	Spermatophore cryopreservation	Fertilisation & hatching rates; post-thaw sperm fertilisation rate	(Chow et al., 1985)
Macrobrachium rosenbergii	Electroejaculation at different time intervals	Spermatophore	4% glutaraldehyde in 0.1 M Cacodylate-buffer	Sperm counts & morphology	Light microscopy & TEM	(Harris and Sandifer, 1986)
Macrobrachium malcolmsonii	Electroejaculation	Spermatophore	Ca^{2+}- free saline	Spermatophore weight, sperm count, % live & abnormal sperm	Spermatophore weight; Trypan blue staining & light microscopy	(Samuel et al., 1999)
Macrobrachium rosenbergii	Electroejaculation	Spermatophore	Glycerol & ethylene glycol in deionizing water	Sperm cryopreservation; artificial insemination	Trypan blue staining & fertilisation rate; light microscopy	(Akarasanon et al., 2004)
Macrobrachium rosenbergii	Post-mortem dissection	Testis, vas deferens	Fixed in Davidson's solution	Spermatogenesis in testis	Light microscopy; SEM; TEM	(Poljaroen et al., 2010)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Macrobrachium rosenbergii	Post-mortem dissection	Androgenic gland	Crustacean physiological saline	Testicular maturation & male sex development factors	Histology; BrdU proliferative cell assay; Immunofluorescent microscopy; ELISA; tissue weight	(Siangcham et al., 2013)
Macrobrachium rosenbergii	Electroejaculation	Sperm	Sterile-filtered pond water	Sperm cryopreservation	Eosin–nigrosin staining; light microscopy; phase contrast microscopy; SEM	(Valentina-Claudet et al., 2016)
Macrobrachium acanthurus	Electroejaculation	Spermatophore	Distilled water	Sperm count, sperm cryopreservation	Eosin-nigrosin staining & light microscopy	(Costa et al., 2017)
Macrobrachium americanum	Electroejaculation	Spermatophore, sperm	Distilled water	Sperm count, sperm viability & normal/abnormal morphology	Eosin-nigrosin staining; Light microscopy; proximate analysis of spermatophores; histology	(Pérez-Rodríguez et al., 2019)
			Marine crab			
Scylla serrata	Post-mortem dissection	Spermatophore & seminal plasma	0.1 M glycine in phosphate buffer saline	Morphology & Cryopreservation of spermatophores, sperm viability assay	Eosin-nigrosin staining	(Jeyalectumie and Subramoniam, 1989)
Scylla serrata	Post-mortem dissection	Spermatophore, sperm	Calcium ionophore A23187 for AR; Ca^{2+}-FASW	Viability of cryopreserved sperm	Artificial induction of AR; hypo/hyperosmotic sensitivity tests for membrane integrity; trypan blue & eosin-nigrosin staining; light microscopy	(Bhavanishankar and Subramoniam, 1997)
Uca pugilator	Post-mortem dissection	Testis	Fixed in Bouin's solution	Testicular maturation index using 5-Hydroxytryptamine (5-HT)	5-HT injection on D1, 5 & 10, then crabs sacrificed on D15.	(Sarojini et al., 1993)
Portunus trituberculatus	Post-mortem dissection	Sperm	Ca^{2+} - FASW	AR, sperm cryopreservation	Calcium ionophore A23187 treatment	(Shuai et al., 2007)
Birgus latro	Post-mortem dissection	Testis, vas deferens	120 min in 20% NaOH	Sperm count	Stereomicroscopy	(Sato et al., 2008)
Perisesarma bidens	Manual removal from the spermatheca	Sperm	Filtered seawater	Artificial insemination	Fertilisation rate	(Sarker et al., 2009)
Scylla serrata	Manual removal from the spermatheca	Spermatophore	Ca^{2+} - FASW	AR	Light microscopy; calcium ionophore A23187 to induce AR *in vitro* assay; TEM	(Zhang et al., 2010)
Charybdis japonica	Post-mortem dissection	Sperm	15% DMSO	Sperm viability, sperm cryopreservation	Light microscopy	(XingHong et al., 2010)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Portunus pelagicus	Post-mortem dissection	Testis, vas deferens	Davidson's fixative; 4% glutaraldehyde & 2% paraformaldehyde in 0.1M PBS (pH 7.4) at 4 °C.	Spermatogenesis (morphology)	Light microscopy & TEM	(Stewart et al., 2010)
Cancer setosus	Post-mortem dissection	Spermatophore	Filtered seawater	Structural & ultrastructural events during the acrosome reaction	Light microscopy & SEM	(Dupré et al., 2012)
Callinectes danae	Post-mortem dissection	Spermatophore, sperm	4% paraformaldehyde	GSI	Histochemistry & light microscopy	(Zara et al., 2012)
Callinectes ornatus	Post-mortem dissection	Testis, vas deferens	4% paraformaldehyde in saltwater	Male reproductive tract histology & histochemistry; GSI	Light microscopy & gonadal weight	(Nascimento and Zara, 2013)
Scylla olivacea	Post-mortem dissection	Sperm	Ringer solution	AR	Eosin-nigrosin staining & light microscopy	(Noorbaiduri et al., 2014)
Portunus armatus	Post-mortem dissection	Whole male reproductive tract	Fixed in Bouin's solution	Male reproductive tract morphology	Light microscopy & TEM	(Ravi et al., 2014)
Charybdis japonica	Manual removal from the spermatheca	Sperm	Ca^{2+}- free saline	Sperm viability, acrosin activity, sperm cryopreservation	Nα-benzol-DL-arginine-p-nitroanilide (BAPNA) substrate method for acrosin activity, eosin B-staining, microscopy; sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)	(Xu et al., 2014)
Scylla tranquebarica	Post-mortem dissection	Sperm	Ca^{2+}- free saline	Sperm count & viability, sperm cryopreservation	Light microscopy	(Fatihah et al., 2018)
Marine lobster						
Homarus americanus	Post-mortem dissection	Testes, vas deferens	Seawater, calcium ionophore A23187 plus 1% DMSO in seawater	AR	Phase contrast microscopy	(Talbot and Chanmanon, 1980)
Homarus americanus	Electroejaculation (12V), spermatophore cut to obtain sperm	Spermatophore	100 µM calcium ionophore A23187	Sperm morphology, AR, vas deferens morphology after extrusion	Light microscopy & TEM	(Kooda-Cisco and Talbot, 1983)
Homarus americanus	Electroejaculation (10mA; 7-8 V; 300-2500 ohms)	Spermatophore	Seawater	Spermatophore quality (presence of sperm), artificial insemination & fertilisation	No. of spermatophores, sperm count & fertilisation rate	(Aiken et al., 1984)
Homarus americanus	Electroejaculation, spermatophores cut in seawater & sperm mass extruded to form suspension	Spermatophore	Seawater, paraffin oil (Fisher, Saybolt viscosity 125/135), 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for EM	Morphology & cryopreservation of spermatophores	Light microscopy & TEM	(Ishida et al., 1986)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Panulirus homarus	Post-mortem dissection	Testis, vas deferens	Davidson's fixative	Male reproductive tract histology & ultrastructure	Light microscopy & TEM	(Lakshmi Pillai et al., 2014)
Panulirus polyphagus	Post-mortem dissection	Sperm	Ca^{2+}- free saline	Sperm counts & viability, sperm cryopreservation	Eosin-nigrosin staining & light microscopy	(Fatihah et al., 2016)
			Marine shrimp			
Penaeus aztecus	Post-mortem dissection	Testes, vas deferens	Karnovsky's fixative	Sperm morphology	Phase contrast microscopy & TEM	(Clark et al., 1973)
Penaeus setiferus; P. stylirostris; P. vannamei;	Electroejaculation	Spermatophore	Direct to female's sternum	Artificial insemination using α-cyanoacrylate adhesive	No. of spermatophores expelled	(Sandifer et al., 1984)
Penaeus setiferus	Electroejaculation	Sperm	Sperm released from sperm mass by glass tissue grinder into Ca^{2+} - free saline (pH 7.4)	Spermatophore weight, sperm count, % live sperm, % abnormal sperm trypan blue staining	Light microscopy	(Leung-Trujillo and Lawrence, 1987)
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+}- FASW	Sperm count, morphology & sperm viability	Spermatophore weight; light microscopy for morphology & count; trypan blue staining; acridine orange fluorescent assays & microscopy in sperm viability; EW-induced sperm reaction	(Wang et al., 1995)
Litopenaeus vannamei	Manual extrusion	Spermatophore	Seawater or Ca^{2+}- free saline	Short-term storage (36h)	Sperm count & morphology using phase-contrast microscopy	(Bray and Lawrence, 1998)
Penaeus setiferus	Electroejaculation with antibiotic after extrusion	Sperm	Ca^{2+}- free saline	Sperm morphology, GSI, sperm count	Trypan blue staining & light microscopy	(Pascual et al., 1998)
Penaeus indicus	Electroejaculation	Spermatophore	Filtered seawater	Cryopreservation of spermatozoa	EW-induced acrosome reaction	(Diwan and Joseph, 1998)
Metapenaeus affinis; Metapenaeus brevicornis	Electroejaculation	Sperm	Not mentioned	Artificial insemination with & without α-cyanoacrylate	No. of spermatophore expelled; spawning & hatching rates;	(Pawar and Mohiuddin, 2000)
Pleoticus muelleri	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Spermatophore weight, sperm count, sperm viability	spermatophore weight; trypan blue staining & light microscopy	(Díz et al., 2001)
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Sperm count & viability	Light microscopy	(Perez-Velazquez et al., 2001)
Trachypenaeus byrdi; Xiphopenaeus riveti; Litopenaeus occidentalis	Manual extrusion	Spermatophore	Filtered natural seawater	AR	EW technique &SEM	(Alfaro et al., 2003)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Spermatophore weight, sperm count, sperm viability	Spermatophore weight; trypan blue staining; light microscopy	(Ceballos-Vázquez et al., 2003)
Litopenaeus vannamei	Manual extrusion	Spermatophore, sperm	Sterile seawater	Sperm, spermatic mass, & whole spermatophore cryopreservation,	Light microscopy; flow cytometry (DNA staining with propidium iodine; PI)	(Lezcano et al., 2004)
Farfantepenaeus paulensis	Manual extrusion	Spermatophore, sperm	Not mentioned	Artificial insemination	Fertilisation rate	(Peixoto et al., 2004a)
Farfantepenaeus paulensis	Manual extrusion	Spermatophore, sperm	Ca^{2+}- free saline	Spermatophore weight, sperm count	Tissue weights & light microscopy	(Peixoto et al., 2004b)
Penaeus monodon	Manual extrusion	Spermatophore	Mineral oil (0.1% penicillin-streptomycin)	Sperm viability, artificial insemination, chilled storage of spermatophore	Eosin–nigrosin staining & light microscopy	(Nimrat et al., 2005)
Penaeus monodon	Manual extrusion	Spermatophore, sperm	5% DMSO + Ca^{2+}- free saline (0.09% NaCl)	Cryopreservation & artificial insemination, sperm count, sperm viability, spermatophore weight, abnormal sperm	Tissue weights; fertilisation rate; light microscopy	(Bart et al., 2006)
Litopenaeus vannamei	Manual extrusion	Spermatophore	Mineral oil with 0.1% penicillin-streptomycin	Sperm viability, chilled storage of sperm	Eosin–nigrosin staining & light microscopy	(Nimrat et al., 2006)
Penaeus monodon	Manual removal from female's thelycum	Spermatophore, sperm	Artificial filtered seawater	Acrosome reaction	EW technique; SEM; TEM	(Pongtippatee et al., 2007)
Penaeus monodon	Manual extrusion	Spermatophore, sperm	Ca^{2+}- free saline	Sperm viability, spermatophore cryopreservation	Eosin-nigrosin staining; light microscopy; fertilisation rate	(Vuthiphandchai et al., 2007)
Farfantepenaeus paulensis	Manual extrusion; electroejaculation	Spermatophore	Ca^{2+}- free saline	Sperm count; spermatophore weight; spermatosomatic index (ESI)	Spermatophore weight & light microscopy	(Nakayama et al., 2008)
Penaeus monodon	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Sperm viability, bacterial profiling for 210 d	Eosin–nigrosin staining; light microscopy; bacterial assay (incl. aseptic techniques)	(Nimrat et al., 2008)
Litopenaeus vannamei	Manual extrusion	Spermatophores	Trypsin at 4 °C for 24 hr, Ca^{2+}-free saline at 25 °C	Sperm count, sperm cryopreservation	Flow cytometry; eosin-nigrosin staining; light microscopy	(Chao et al., 2009)
Farfantepenaeus paulensis	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Spermatophore weight, sperm count, melanisation & spermatophore absence rates	Spermatophore weight & light microscopy	(Braga et al., 2010)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Litopenaeus vannamei	Manual extrusion	Spermatophore	2.5% glutaraldehyde in artificial seawater (pH 8.0); 4% paraformaldehyde	Ultrastructure & biochemistry of sperm capacitation	EM/LSCM; confocal immunofluorescent microscopy to detect tyrosine-phosphorylated proteins	(Aungsuchawan et al., 2011)
Rhynchocinetes typus	Post-mortem dissection; electroejaculation	Vas deferens	Filtered seawater	Gamete & zygote morphology during fertilisation	Light microscopy; confocal fluorescent microscopy; SEM; TEM	(Dupré and Barros, 2011)
Penaeus merguiensis	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Sperm viability, spermatophore cryopreservation	Eosin-nigrosin staining; light microscopy; fertilisation rate	(Memon et al., 2012)
Litopenaeus vannamei	Manual extrusion	Sperm	Modified artificial saline (antibiotic/antimycotic, pH 7.4)	Short – term storage of sperm, artificial insemination	Fertilisation rate	(Morales-Ueno et al., 2013)
Litopenaeus vannamei	Manual extrusion	Spermatophore, sperm	Ca^{2+}- free saline	GSI, spermatophore energy levels & sperm count	Trypan blue exclusion test; light microscopy; sex organ weight	(Vázquez-Islas et al., 2013)
Stenopus hispidus	Post-mortem dissection	Whole reproductive tract	2.5% glutaraldehyde & 4% paraformaldehyde in 0.1 mol L^{-1} phosphate buffer (pH 7.3; Karnovsky solution)	Male reproductive tract morphology	SEM	(Gregati et al., 2014)
Litopenaeus schmitti	Post-mortem dissection	Spermatophore	Ca^{2+}- free saline	Sperm cryopreservation, sperm count	Eosin-nigrosin staining & light microscopy	(Chaves et al., 2014)
Farfantepenaeus paulensis	Manual extrusion	Sperm	Ringer solution	Sperm count, DNA damage	Eosin-nigrosin staining, SEM; Comet assay	(Noor-Hidayati et al., 2014)
Litopenaeus vannamei	Manual extrusion	Spermatophore	Sterile seawater; Freezing solution (5% egg yolk & 0.2 M sucrose)	Sperm count, sperm cryopreservation	Eosin-nigrosin staining; light microscopy; flow cytometry	(Uberti et al., 2014)
Litopenaeus vannamei	Manual extrusion	Sperm	Ca^{2+}- free saline	Sperm viability, sperm cryopreservation	Eosin–nigrosin staining; 6-carboxyfluorescein diacetate (CFDA) & PI staining; fluorescent microscopy	(Castelo-Branco et al., 2015)
Litopenaeus vannamei; Farfantepenaeus; subtili; Litopenaeus schmitti	Manual extrusion	Sperm	Ca^{2+} - FASW	Spermatophore weight, sperm count & sperm viability	Light microscopy; fluorescence microscopy	(Silva et al., 2015)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	Reference
Penaeus monodon	Post-mortem dissection	testis	Davison's solution; 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate with 5% sucrose (pH 7.4)	Spermatogenesis (morphology)	Light microscopy & TEM	(Feng et al., 2017)
Litopenaeus vannamei	Manual extrusion	Spermatophore	LN_2 for vitrification Ca^{2+} - free saline & 0.4 M trehalose	Sperm vitrification, sperm count, artificial insemination, membrane integrity	Fluorescent microscopy & light microscopy	(Castelo-Branco et al., 2018)
Palaemon serratus	Manual extrusion	Sperm	Artificial seawater	DNA integrity	Comet Assay	(Erraud et al., 2018)
Penaeus monodon	Post-mortem dissection	Reproductive organ	Davidson's solution	Acrosome formation & spermatophore formation	Histology; light microscopy; TEM	(Feng et al., 2018b)
Penaeus monodon	Electroejaculation	Spermatophore	Ca^{2+}- free saline	DNA fragmentation	Comet assay, sperm chromatin dispersion test (SCDt)	(Feng et al., 2018a)
Fenneropenaeus indicus	Manual extrusion	Sperm	Ca^{2+}- free saline	Sperm viability, sperm cryopreservation	Eosin-nigrosin staining; light microscopy; Hypo – osmotic swelling test (HOST) & DNA integrity analyses; fluorescent microscopy.	(Selvakumar et al., 2018)
Penaeus monodon	Electroejaculation	Spermatophore	Artificial lobster haemolymph (AH), Ca^{2+}- free saline & Ca^{2+} - FASW	Plasma membrane integrity, % AR & % sperm DNA fragmentation (SDF) after 4 °C storage for 0 – 26 d.	AR assay *in vitro*; Sperm viability assay; Fluorescent microscopy	(Feng et al., 2019)

Preparation of a single-cell suspension of spermatozoa is often a prerequisite for sperm quality evaluation. In decapod crustaceans, a single-cell suspension of spermatozoa can be achieved mechanically by gentle homogenisation of the spermatophore using a tissue grinder, or by vigorous repeated pipetting in a semen extender solution to disrupt the walls of the spermatophore (Leung-Trujillo and Lawrence, 1987; Bugnot and López Greco, 2009a; Gwo, 2009; Feng et al., 2019). Extracted sperm cells are then filtered to remove debris and pelleted by centrifugation at 200 – 500 x *g* for 5 min. Sperm cells can survive a series of resuspension and centrifugation steps during the washing process (Gwo, 2009). Spermatozoa can also be extracted from the spermatophore by chemical treatments such as trypsin (Chao et al., 2009; Wang et al., 2015) or pronase digestion (Bhavanishankar and Subramoniam, 1997) at 4 ºC for a period of time prior to mechanical homogenisation in semen extender. However, one must be careful with such treatments since pronase is known to induce acrosome reaction in spermatozoa, which should be avoided to prolong short-term sperm storage (Bhavanishankar and Subramoniam, 1997).

Once spermatophores are extruded, they are often held in a semen extender solution prior to sperm quality assessment (Van Harreveld, 1936; Morales-Ueno et al., 2013). A semen extender is typically a physiological saline solution made of salts and sugars, which is added to the seminal fluid to prolong sperm viability after the collection of extruded spermatozoa (Morales-Ueno et al., 2013; Chapman, 2016). One critical factor for a good semen extender is its ability to prevent functional activation during the collection, handling, and storage of spermatozoa (Park and Chapman, 2005; Cloud and Patton, 2009; Chapman, 2016). It should also provide an isotonic environment for spermatozoa with good pH buffering capacity, and include nutrients and sugars necessary for sperm cells survival, as well as antioxidants to

control reactive oxygen species and antibacterial substances to fight bacterial proliferation (Mansour et al., 2004; Park and Chapman, 2005).

While long-term cryopreservation of spermatozoa is discussed further in section 2.4, short-term storage of both spermatophore and spermatozoa helps enhance reproductive management at the hatchery by allowing more time to complete sperm quality assessments and adding more flexibility to carry out breeding programs, specifically for artificial insemination (Chow, 1982; Talbot et al., 1986; Bart et al., 2006), where spermatozoa need to be maintained *in vitro* for a short period prior to the insemination procedure (Morales-Ueno et al., 2013). By developing an optimised semen extender, the number of females inseminated per male can be maximised while the time window to execute artificial insemination can be increased.

In *Penaeus* shrimp, a species-specific artificial semen extender solution was developed for whiteleg shrimp consisting of 2.125 g. L^{-1} NaCl, 0.110 g. L^{-1} KCl, 0.052 g. L^{-1} H_3BO_3, 0.019 g. L^{-1} NaOH, and 0.484 g. L^{-1} $MgSO_4{\cdot}7H_2O$ with 20 µL of antibiotic/antimycotic (10,000 U Penicillin, 10 mg Streptomycin, 25 µg Amphotericin B) at pH 7.4. This extender reportedly facilitated a > 60 % fertilisation rate following artificial insemination (Morales-Ueno et al., 2013). Whiteleg shrimp spermatophores stored in this extender can be maintained for up to 26 h at 14 ºC, with 92 ± 15 % spermatozoa showing normal morphology (Morales-Ueno et al., 2016). For decapods, Ca^{2+}-free saline is normally recommended as a semen extender during sperm cryopreservation since the absence of calcium prevents the initiation of the acrosome reaction in spermatozoa (Bart and Dunham, 1996; Vuthiphandchai et al., 2007; Feng et al., 2019). Ca^{2+}-free saline is composed of 21.63 g. L^{-1} NaCl, 1.12 g. L^{-1} KCl, 0.53 g. L^{-1} H_3BO_3, 0.19 g. L^{-1} NaOH, 4.93 g. L^{-1} $MgSO_4{\cdot}7H_20$ at pH 7.5 (Vuthiphandchai et al.,

2007). A list of different semen extender solutions and sperm quality assessments for crustaceans is shown in Table 2.1; they include physiological saline for freshwater crustaceans; filtered natural or artificial seawater, or mineral oil for marine crustaceans. Meanwhile, Ca^{2+}-free saline or Ringer's and phosphate-buffered saline solutions have been used for both marine and freshwater crustaceans.

2.3.2. Advanced tools for sperm quality evaluation

The ultimate measures of male fertility in crustaceans are fertilisation and hatching rates. However, these measures take time to properly confirm, require the simultaneous availability of eggs to conduct the tests, and can also be significantly affected by female factors such as egg/embryo quality and culture conditions, which may yield variable results. Thus, assessment of male fertility by direct evaluation of sperm quality is a more feasible alternative since it can rapidly and objectively detect deleterious effects caused by poor male health, nutrition, husbandry, genetics, or other factors (Rurangwa et al., 2004).

2.3.2.a. Sperm number, morphology, and membrane integrity

Once the spermatophore is collected and is allowed to soften in physiological saline for crustaceans, a single-cell suspension of spermatozoa can be prepared by gentle agitation, which then permits sperm number to be quantified (Van Harreveld, 1936; López Greco and Lo Nostro, 2008; Bugnot and López Greco, 2009a, 2009b; Harlioğlu et al., 2012; Harlioğlu et al., 2013). Generally, greater numbers of spermatozoa are associated with greater reproductive capacity among males of most species (Bart and Dunham, 1996; Rurangwa et al., 1998; Liley et al., 2002; Rurangwa et al., 2004; Bombardelli et al., 2013). In freshwater

crayfish, sperm number is determined using a Neubauer haemocytometer and is typically expressed as the number of sperm/DVD section. In male redclaws weighing 70 – 110 g, sperm concentration ranges from 10^8 to 10^9 sperm/l cm DVD section (Bugnot and López Greco, 2009a, 2009b). Typically, decapod spermatozoa are non-motile and without flagella (Beach and Talbot, 1987; Kouba et al., 2015; Subramoniam, 2017a, 2017c). Their spermatozoa are generally composed of a main body enclosing a decondensed nucleus, a highly complex acrosome in the anterior region, and no discernible midpiece (compared to mammalian sperm). From the main body, different numbers of stellate processes that project outside arise, but almost in a species-specific manner (Lewis and Ford, 2012; Subramoniam, 2017a). Thus, assessing sperm quality using simple light microscopy is difficult because distinct morphological features are lacking (Lezcano et al., 2004). Moreover, sperm motility, a good biomarker to assess competitive swimming ability to fertilise eggs during spawning in finfish and other species, cannot be used for these crustaceans (Lezcano et al., 2004). As such, researchers tended to use higher magnification microscopy, such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), to study decapod sperm morphology (Yasuzumi and Lee, 1966; Anderson and Ellis, 1967; Beach and Talbot, 1987; An et al., 2011; Niksirat et al., 2013; Kouba et al., 2015), which is time-consuming, costly, and limited to low numbers of cells and individuals that can be evaluated. Thus, the need to assess decapod spermatozoa more rapidly has stimulated the adoption of newer methods (Lezcano et al., 2004; Lewis and Ford, 2012).

Abnormal sperm morphology can also be an indicator of infertility or aberrant spermatogenesis (Leung-Trujillo and Lawrence, 1987; Meunpol et al., 2005; Leelatanawit et al., 2014; Harlıoğlu et al., 2018). In decapod crustaceans, live spermatozoa can be examined by light microscopy for sperm abnormalities such as deformed or missing heads, twisted or

missing spikes, and distorted main bodies (Leung-Trujillo and Lawrence, 1987; Leelatanawit et al., 2014; Harlıoğlu et al., 2018). However, the absence of distinct morphological structures in mature decapod spermatozoa, such as a lack of flagella or few or absent mitochondria, makes the rapid assessment of normal *vs.* abnormal morphology by light microscopy more difficult (Beach and Talbot, 1987; Lezcano et al., 2004; Kouba et al., 2015).

Given the limitations mentioned above, alternative approaches have been considered, such as evaluating the integrity of the plasma membrane to assess sperm viability (Friend and Rudolf, 1974; Evenson et al., 1982; Gledhill, 1983; Peña et al., 1998; Niksirat et al., 2014b; Hirohashi and Yanagimachi, 2018). For sperm viability in decapod crustaceans, spermatozoa are typically stained with conventional (Trypan blue or eosin-nigrosin) or fluorescent (propidium iodide, PI) exclusion dyes in Ca^{2+} - free saline (pH 7.4) to examine the integrity of the cell's plasma membrane to determine the proportion of live (unstained) *vs.* dead (stained) spermatozoa (Talbot and Chacon, 1981; Leung-Trujillo and Lawrence, 1987; Cabrita et al., 2009; Lewis and Ford, 2012; Leelatanawit et al., 2014; Selvakumar et al., 2018). The presence of calcium in semen extenders can trigger the acrosome reaction (Tereza and Halcrow, 1988; Lindsay and Jr., 1992; Breitbart, 2002). Thus, Ca^{2+}-free saline is generally used as the semen extender in most sperm viability studies because the absence of calcium prevents the acrosome reaction and subsequent rupture of the sperm plasma membrane (Bart and Dunham, 1996; Vuthiphandchai et al., 2007; Feng et al., 2019). Cells treated with conventional stains require light microscopy to detect and count dead cells manually; which can be labour-intensive, permitting typically only ~200 cells per individual to be examined (Leung-Trujillo and Lawrence, 1987; Lezcano et al., 2004; Gillan et al., 2005). By contrast, treating cells with structure-specific fluorescent stains enables the rapid analysis of more than

20,000 cells per individual to be examined in a matter of minutes using high-throughput flow cytometry (Garner et al., 1986; Gillan et al., 2005; Liu et al., 2007; Peña et al., 2019b).

When using flow cytometry, spermatozoa are often counterstained with carboxyfluorescein diacetate (CFDA), SYBR®-14, or Hoechst 33342 (Garner et al., 1986; Garner and Johnson, 1995; Kordan et al., 2013). CFDA binds to esterases, which stains live spermatozoa with intact cell membranes fluorescent green; while SYBR®-14 and Hoechst 33342 bind to nucleic acids, which stains the nucleus of live spermatozoa green and blue respectively (Kordan et al., 2013; Silva et al., 2015). By contrast, PI binds to nucleic acids but can only penetrate cells that have ruptured membranes, causing the nucleus of dead spermatozoa to stain fluorescent red (Garner and Johnson, 1995; Kordan et al., 2013; Silva et al., 2015). In crustaceans, fluorescent dyes coupled with flow cytometry showed 33 – 89% post-thaw viability in whiteleg shrimp spermatozoa after exposure to different cryopreservation protocols (Lezcano et al., 2004; Chao et al., 2009; Uberti et al., 2014). In freshwater crabs, PI staining and flow cytometry showed that sperm plasma membranes were significantly damaged (17 – 20 % dead spermatozoa) when exposed to high concentrations of lead (Li et al., 2016). These results show that fluorescent stains coupled with flow cytometry represent a sensitive tool for assessing sperm cell viability in decapod crustaceans.

2.3.2.b. Acrosome reaction

The acrosome reaction (AR) of spermatozoa involves the fusion of the outer acrosomal membrane with the overlying sperm plasma membrane to permit a spermatozoon to penetrate the outer membrane of the egg and, by so doing, deliver paternal genetic material into the egg that is required for successful fertilisation (Kang et al., 2009). AR in decapod spermatozoa

involves molecular and morphological changes to the acrosomal vesicle and the introduction of subacrosomal and nuclear materials into the egg (Talbot and Chanmanon, 1980). AR is a reliable predictive biomarker of sperm quality, especially for non-motile spermatozoa often found in decapod crustaceans (Kang et al., 2009; Farhadi, A. and Harlıoğlu, A.G., 2019). However, infertile males can have damaged/absent acrosomes, thereby preventing such spermatozoa from fertilising eggs. In addition, various external stressors during semen processing and/or freezing can induce damage to the acrosome membrane or provoke a premature acrosome reaction, leading to misleading infertility diagnoses that need to be carefully controlled (Parinaud et al., 1996; Tello-Mora et al., 2018).

AR has been well-studied in decapod crustaceans including crabs, lobsters and Penaeid shrimp (Pongtippatee et al., 2007; Kang et al., 2009; Zhang et al., 2010; Dupré et al., 2012; Noorbaiduri et al., 2014; Wang et al., 2015; Farhadi, A. and Harlıoğlu, A.G., 2019). Briefly, the AR can be induced by suspending spermatozoa in physiological saline that has been used to incubate eggs *in vitro* (i.e., egg water; EW). Alternately, AR can be observed naturally at the oviposition of a copulated female, which involves the immediate collection of eggs and spermatozoa from the female's seminal receptacle (Pongtippatee et al., 2007).

For decapod sperm, the capability to undergo AR is considered a good biomarker of normal acrosome functionality (Wang et al., 1995; Feng et al., 2019). The AR in decapods can be induced in several ways, including exposure to egg water, calcium ionophore, alkalinisation, high concentrations of ions, and cold shock (Lindsay and Jr., 1992; Kang et al., 2009; Zhang et al., 2010; Chávez et al., 2018; Farhadi, A. and Harlıoğlu, A.G., 2019). Egg water, which is commonly used for AR induction *in vitro* (Pongtippatee et al., 2007; Kruevaisayawan et al., 2008), typically contains vitelline envelope (VE), the external layer of eggs, cortical rods

(CRs; egg jelly material located in egg surface crypts), and some thelycal (T) substances. (Clark Jr and Griffin, 1988; Griffin and Clark Jr., 1990; Pongtippatee et al., 2007; Kruevaisayawan et al., 2008). Egg water has approximately a 4:1 protein-to-carbohydrate ratio and contains trypsin-like enzymes, which are natural inducers that play a crucial role during the second stage of AR in decapods (Griffin and Clark Jr., 1990; Kruevaisayawan et al., 2008). Calcium ionophore A23187 can also induce the AR in decapod spermatozoa by increasing calcium influx into the cells (Clark Jr and Griffin, 1988; Lindsay and Jr., 1992). In the freshwater Chinese mitten crab (*Eriocheir sinensis*), AR reportedly can be induced by egg water, seawater, $CaCl_2$ solution or low temperature (Nanshan and Luzheng, 1987; Li et al., 2010). In addition, cryopreservation can also trigger AR in spermatozoa of *E. sinensis* (Kang et al., 2009) by directly promoting membrane fusion of the acrosomal cap or by the destruction of AR inhibiting and activation of AR-promoting proteins (Okada et al., 2001; Kang et al., 2009).

Presently, efforts to quantify acrosome reaction in decapod crustaceans involve counting of acrosome-reacted *vs.* normal intact spermatozoa based on their morphological appearance using light microscopy (Kang et al., 2009; Feng et al., 2019). However, this requires considerable familiarity with acrosome morphology and the mechanism of AR for the decapod species of interest (Talbot and Chanmanon, 1980; Pongtippatee et al., 2007; Kang et al., 2009; Braga et al., 2013). Moreover, some decapods (infraorder *Caridea*) appear to lack acrosome-like structures precluding the evaluation of the AR in these species (Braga et al., 2013). By contrast, acrosome-specific biostaining techniques using fluorescein isothiocyanate (FITC)-conjugated *Arachis hypogaea* (peanut) lectin (FITC-PNA) can be employed to analyse the acrosome reaction across multiple decapod groups. The PNA lectin is specific for terminal β-galactose moieties and so will bind to the acrosome in acrosome-reacted sperm,

fluoresce green (Ashizawa et al., 2006; Lewis and Ford, 2012), and can be quantified via fluorescent microscopy or flow cytometry without the need to understand species-specific acrosome morphologies (Graham et al., 1990; Favret and Lynn, 2010; Hossain et al., 2011).

2.3.2.c. Mitochondrial function

An array of fluorescent dyes can be used as biomarkers for other specific sperm functions. Mitochondria are the source of ATP production in most eukaryotic cells, which is the basic unit of energy used to power their function (Kowaltowski et al., 2009). Several dyes have been employed to assess mitochondrial membrane integrity and functionality. For example, mitochondrial stain, MitoTracker-Red CMXRos (M-7512) has been used to stain functional mitochondria fluorescent red in viable cells with dye accumulation due to mitochondrial membrane potential (Lewis and Ford, 2012). However, the most common combination of dyes used in aquaculture species is dual staining using rhodamine 123 (R123) and PI. Spermatozoa with intact plasma membrane and functional mitochondria take up R123 and fluoresce green, while PI stains the nucleus of dead cells with damaged membranes fluorescent red (Garner et al., 1986; Garner et al., 1997). In aquaculture, the R123/PI staining method coupled with flow cytometry has been used to evaluate mitochondrial function in both marine invertebrates (Adams et al., 2003) and finfishes (De Baulny et al., 1997; Ogier de Baulny et al., 1999; Liu et al., 2007). The mitochondrial stain carbocyanine fluorescent probe, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetrathylbenzimidazolyl-carbocyanine iodide (JC-1) is another widespread fluorochrome used to assess changes in mitochondrial membrane potential in mammalian sperm (Gravance et al., 2000; Martinez-Pastor et al., 2004; Nesci et al., 2020). JC-1 can distinguish between spermatozoa with high *vs.* low functional mitochondria (Gillan et al., 2005). JC-1-stained spermatozoa fluoresce green when

mitochondrial function is low; fluoresce orange/green when mitochondrial function is high; and fluoresce red when mitochondrial function is extremely high (Reers et al., 1995; Cossarizza and Salvioli, 2000; Gillan et al., 2005; Binet et al., 2014). The protonophore, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) impedes mitochondrial function by uncoupling oxidative phosphorylation and has been employed as a positive control for various cell types stained with JC-1 including both marine invertebrates (Binet et al., 2014; Boulais et al., 2015) and finfish (Guthrie et al., 2008) spermatozoa.

Presently, there are no reports documenting mitochondrial function in decapod crustaceans. This may be related to the observation that mitochondria in mature decapod spermatozoa appear to be degenerate, transformed, non-christate, non-functional, or even absent in some species (Anderson and Ellis, 1967; Braga et al., 2013; Feng et al., 2017; Subramoniam, 2017a). In some decapods, such as freshwater crayfish, few mitochondria have been observed to be associated with the membrane lamellae complex (Beach and Talbot, 1987; Kouba et al., 2015). Mitochondria in this taxonomic group do not generate energy, but Anderson and Ellis (1967) have reported that the membrane lamellae complex is responsible for ATP production in mature sperm of crayfish (Anderson and Ellis, 1967). In the Danube crayfish (*Astacus leptodactylus*), the membrane lamellae complex was also reported to separate from the cell after discharge of spermatozoa from the spermatophore (Niksirat et al., 2014b). Given spermatozoa of most species of decapods are immotile, and few studies have reported functional mitochondria, the role of sperm mitochondria in male fertility may be relatively limited compared to fish and mammalian counterparts. However, the above assays may still help facilitate a greater understanding of the function of the membrane lamellae complex as an energy source and the role (if any) of mitochondria during fertilisation in mature sperm of decapod crustaceans.

2.3.2.d. Sperm DNA fragmentation

Sperm DNA plays a crucial role in facilitating normal embryo development and live birth (Dar et al., 2013; Peña et al., 2017; Figueroa et al., 2020). As such, DNA is highly condensed and efficiently packed in spermatozoa to avoid damage during transport to the site of fertilisation (Peña et al., 2017; Panner Selvam and Agarwal, 2018; Figueroa et al., 2020). The sperm DNA is wrapped around histone proteins, which during spermatogenesis, are gradually replaced by highly basic protamines that facilitate greater condensation (Peña et al., 2017; Panner Selvam and Agarwal, 2018; Figueroa et al., 2020). During this process, transcription and translational of sperm DNA ceases. Moreover, during condensation, double-stranded DNA incurs torsional stress resulting in nicks and breaks along the DNA strand (Panner Selvam and Agarwal, 2018; Figueroa et al., 2020). Failure to repair these nicks and breaks, combined with the cumulative effect of reduced protamination, could lead to DNA damage (Bungum et al., 2011; Peña et al., 2017; Panner Selvam and Agarwal, 2018; Figueroa et al., 2020).

Interestingly, decapod spermatozoa are composed of a main body that envelops a decondensed nucleus of chromatin fibres (Poljaroen et al., 2010; Kouba et al., 2015; Feng et al., 2017), where histones are relatively low to nil and protamines are completely absent (Sellos and Legal, 1981; Kurtz et al., 2009; Poljaroen et al., 2010; Feng, 2018). Spermatozoa with poor chromatin packaging and low protamine content are susceptible to oxidative stress (imbalance between oxidation and reduction reactions; Wagner et al., 2018). Decondensed nuclei composed of diffuse and heterogeneous chromatin fibres in decapod crustacean sperm can be highly susceptible to DNA damage (Aitken et al., 2004; Zhao et al., 2019). The reactive oxygen species (ROS) generated by oxidative stress are an intrinsic source of sperm

DNA damage (Agarwal et al., 2017; Wagner et al., 2018). Oxidative stress is caused by insufficient antioxidants to neutralise free radicals generated during spermatogenesis. ROS attacks spermatozoa during spermatogenesis by activating endonucleases or caspases that cause DNA damage. Caspases are enzymes directly involved in DNA fragmentation and cell death (Wagner et al., 2018). In contrast to egg cells, spermatozoa lack the ability to prevent and repair DNA damage induced by environmental stressors (Aitken et al., 2004; Erraud et al., 2018; Zhao et al., 2019). Oxidative stress can also result in lipid peroxidation, protein alterations, and sperm apoptosis, which further compromise the paternal DNA contributed to the developing embryo (Aitken et al., 2012; Wagner et al., 2018; Figueroa et al., 2019; Figueroa et al., 2020). Increased DNA damage in decapod spermatozoa has been reported when exposed to pollutants, such as heavy metals (Yang et al., 2008; Li et al., 2016; Zhao et al., 2019), and environmental stressors, including low temperature (Qiu et al., 2011) and extremities in pH (Wang et al., 2009).

Recently in vertebrates, supplementation of antioxidants either in the diet or semen extender reduces the effects of oxidative stress on DNA integrity, mitigating the effect of ROS (Figueroa et al., 2017; Figueroa et al., 2018; Peña et al., 2019a; Peña et al., 2019b; Figueroa et al., 2020). For example, the use of antioxidants such as α-tocopherol and ascorbic acid in sperm freezing medium reduced lipid peroxidation and increased fertilisation rate (80 – 90%) in cryopreserved spermatozoa of Atlantic salmon (Figueroa et al., 2018). Alternatively, antioxidant supplementation of boar diets resulted in a 55% reduction in sperm DNA damage induced by heat-stress (Peña et al., 2019a; Peña et al., 2019b). To date, the use of antioxidants to improve sperm quality in decapod crustaceans has not been reported, warranting further investigation.

Sperm DNA damage is a crucial indicator of male infertility (Zheng et al., 2018). Sperm with DNA damage may look healthy when using traditional measures of assessing sperm quality and can still fertilise oocytes (Ahmadi and Ng, 1999; Devaux et al., 2011; Castelo-Branco et al., 2018; Fernández-Díez and Herráez, 2018; Erraud et al., 2019). However, structural damage in DNA can lead to abnormalities in pronuclear formation, activation of key embryonic genes and early embryo development (Evenson, 1999; Peña et al., 2017). During embryo development, the first 2-cell divisions are primarily controlled by maternal reserves of proteins and enzymes accumulated in the egg, but these need to be replenished by activation of the embryonic genome (containing both maternal and paternal DNA) from around the 4-cell stage in most species (Braude et al., 1988). High levels of sperm DNA damage can induce delayed embryo cleavage, abnormal embryo morphology, and lower rates of blastocyst formation and implantation (Parinaud et al., 1993; Janny and Menezo, 1994; Paul et al., 2008; Dar et al., 2013).

DNA damage has been reported to occur in the spermatozoa of vertebrates and invertebrates, including crustaceans (Erraud et al., 2018; Feng et al., 2018a; Erraud et al., 2019). For example, in whiteleg shrimp, sperm DNA damage was thought to be the cause of low to zero hatching rates after artificial insemination, despite good rates of egg fertilisation (Castelo-Branco et al., 2018). Chinese freshwater crabs (*Sinopotamon henanense*) exposed to higher concentrations of various heavy metals exhibited poor sperm quality with a high proportion of DNA fragmentation (Wang et al., 2011; Ma et al., 2013; Li et al., 2016). Although conventional sperm analyses such as sperm counting and spermatophore weight can be used to characterise sperm quality, molecular assays such as sperm DNA fragmentation (SDF) analysis, can provide insight into the developmental competence of embryos fertilised by a male's sperm, and hence, a more accurate measure of fecundity (Peña et al., 2017; Zheng et

al., 2018; Peña et al., 2019a; Peña et al., 2019b). Thus, the development of sperm DNA damage assays for decapod crustaceans could identify putative causes of poor juvenile production yields in commercial crustacean aquaculture.

Several assays have been developed to measure sperm DNA damage, including traditional staining methods, toluidine blue staining, sperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD) or Halosperm test, terminal deoxynucleotidyl transferased dUTP nick-end labelling (TUNEL), and the Comet assay directly or indirectly (Panner Selvam and Agarwal, 2018). However, given their different mechanisms for detecting DNA damage, the results from each test are unique and not comparable (Simon et al., 2014; Peña et al., 2017; Peña et al., 2019b). Given that sperm DNA damage can increase significantly after prolonged storage or incubation during sample preparation (Nabi et al., 2014), spermatozoa should be fixed shortly after collection and then either: i) smeared, air-dried, and stained on glass slides before analysis by light/fluorescent microscopy, or ii) washed, permeabilised and stained before analysis by flow cytometry (Nabi et al., 2014).

Toluidine blue staining is used to evaluate damage to the nuclear chromatin structure of spermatozoa and is visualised by light microscopy (Erenpreisa et al., 2003; Panner Selvam and Agarwal, 2018). Using toluidine blue, sperm heads with high chromatin integrity stain blue, while sperm heads with damaged chromatin stain purple (Erenpreisa et al., 2003). By contrast, chromomysin A3 (CMA3) dye preferentially binds to spermatozoa with protamine deficiency, indicating that DNA is poorly packed or damaged (Manicardi et al., 2011; Zandemami et al., 2012). As a result, faint yellow CMA3 dye staining indicates spermatozoa with normal or high protamination, whereas bright yellow staining indicates protamine

deficiency associated with high DNA damage (Manicardi et al., 1995; Zandemami et al., 2012).

The sperm chromatin structure assay (SCSA) is commonly used to detect sperm DNA damage in vertebrates (Gillan et al., 2005; Panner Selvam and Agarwal, 2018). It is an indirect assay in which DNA is denatured by heat or acid treatment, causing single-stranded DNA breaks followed by AO staining (Darżynkiewicz et al., 1975). AO binds to intact double-stranded DNA or denatured ssDNA to exhibit intense green or red fluorescence, respectively, which can be further assessed through a flow cytometer (Darżynkiewicz et al., 1975; Panner Selvam and Agarwal, 2018). This assay can evaluate fresh and frozen samples with high repeatability and less inter- and intra-sample variability (Bungum et al., 2011; Evenson, 2016; Cho and Agarwal, 2017; Panner Selvam and Agarwal, 2018). However, so far, there is no report on the use of SCSA in fish or crustaceans (Cabrita et al., 2014).

Sperm chromatin dispersion (SCD) or Halosperm test is another indirect assay to evaluate sperm DNA damage. For SCD assay, spermatozoa are embedded in agarose on a slide, and their DNA is denatured with an acid solution to yield halos or dispersed chromatin due to relaxed DNA, which can be visualised by fluorescent microscopy (Panner Selvam and Agarwal, 2018). Spermatozoa with fragmented DNA produce small or no halos of dispersed DNA, while spermatozoa with intact DNA produce medium to large halos of DNA (Fernández et al., 2003; Fernández et al., 2005; Tandara et al., 2014). In the black tiger prawn, this assay found greater sperm DNA fragmentation in domestic *vs.* wild-caught individuals ($6.8 \pm 4.5\%$ *vs.* $3.3 \pm 1.5\%$; $n = 10$; Feng et al., 2018a). Thus, the SCD test could be a reliable predictive biomarker to assess male fertility for broodstock management in saltwater prawn aquaculture (Feng et al., 2018a).

The comet assay, an alkaline version of single-cell gel electrophoresis, is a test in which spermatozoa are embedded in agarose on a slide, lysed with detergent to release DNA, and then subjected to electrophoresis before DNA is stained by SYBR Green I for visualisation using fluorescent microscopy (Feng et al., 2018a). During electrophoresis, small, fragmented DNA strands migrate through the agarose away from the nucleus and are visualised as a 'comet-like' tail, while larger intact DNA remains compact in the sperm head. The length of migration of the tail and the intensity of fluorescent green staining are directly proportional to the amount of DNA damage within each spermatozoon (Simon and Carrell, 2013). While the comet assay can detect many types of DNA fragmentation, it is effective only for fresh samples and analyse only a few cells (Jackson et al., 2013; Hobbs et al., 2020). The Comet assay has been used to evaluate DNA fragmentation in crustacean spermatozoa. In black tiger prawns, a two-tail comet assay was performed to validate sperm nuclear morphologies, and % SDF was qualitatively determined using the SCD test (Feng et al., 2018a). Erraud et al. (2018) utilised the comet assay to determine declines in sperm quality among palaemonid prawns exposed to contamination; subsequently recommending its use as a potential predictive marker for *in situ* biomonitoring surveys (Erraud et al., 2018).

While the comet assay relies on fluorescent microscopy and only works for fresh samples, the TUNEL assay can utilise either fluorescent microscopy or flow cytometry on fresh, fixed or cryopreserved samples (Bungum et al., 2011). TUNEL is a direct assay that targets DNA strand breaks by incorporating fluorescein isothiocyanate (FITC) conjugated 2'-deoxyuridine 5'-triphosphates (dUTPs) to the 3'hydroxyl (OH) breaks of single-stranded and double-stranded DNA. As such, the nucleus of DNA-damaged cells fluoresces green. Either PI or Hoechst 33342 dyes can be used as nucleic acid counterstain and fluoresce red or blue,

respectively (Panner Selvam and Agarwal, 2018; Van den Berghe et al., 2018). TUNEL has been used in a limited number of crustacean studies to assess DNA fragmentation, for example, DNA damage caused by viral diseases in black tiger prawns (Sahtout et al., 2001; Kornnika et al., 2002; Kanokpan et al., 2003) during the immune response of *Marsupenaeus japonicus* (Shu and Zhang, 2017), and during neurogenesis of *Homarus americanus* (Harzsch et al., 1999).

In cryopreserved boar, human, mouse, and fish spermatozoa, DNA damage and alterations in downstream expression of specific genes involved in embryo development have been observed (Ron-El et al., 1991; Zilli et al., 2003; Fraser and Strzezek, 2004; Yildiz et al., 2007; Fernández-Díez and Herráez, 2018). Sperm DNA is sensitive to external stressors such as radiation, toxins, or temperature during spermiogenesis, causing oxidative stress-induced genomic lesions. (Pérez-Cerezales et al., 2009; Figueroa et al., 2020). Recently, sperm DNA integrity has been evaluated by examining genomic stability and expression of growth-related genes during embryonic development (Figueroa et al., 2020). Modern technologies such as microarray analysis, quantitative real-time PCR analysis (qRT-PCR), next-generation sequencing (NGS) and bioinformatics have been used to assess variation in sperm genomic DNA and gene expression in aquatic vertebrates such as finfishes (Bäumer et al., 2018; Fernández-Díez and Herráez, 2018; Figueroa et al., 2020). Although the use of these more recent genetic technologies has not been documented in decapod crustaceans, the investigation of sperm DNA damage and downstream alteration of gene expression during early and late embryo development could be of value, particularly to burrowing decapods that may be exposed to heavy metals or toxins in the sediment of aquaculture ponds or in their natural habitat.

The development of sperm quality assays aids the selection of high-quality male broodstock and allows for the production of genetically superior offspring via natural and artificial breeding programmes. Such diagnostic tools could also be used to assess post-thaw sperm quality during the development of cryopreservation and artificial fertilisation protocols for decapod crustaceans. A summary of traditional and advanced biomarkers of sperm quality and conditions that may improve male reproduction management is illustrated in Figure 2.1.

2.4. Sperm cryopreservation

Cryopreservation of crustacean sperm can facilitate the preservation and collection of high-quality male genetic material. This method permits frozen-thawed spermatozoa to be readily available to fertilise eggs whenever they are released, thus allowing powerful control of the timing of reproduction (Cabrita et al., 2014). In addition, frozen spermatozoa can serve as a safe backup in situations where it is difficult to obtain fresh spermatophores, or there is an insufficient number of spermatozoa available for downstream applications like artificial fertilisation (Cabrita et al., 2010). Finally, sperm freezing can preserve the germplasm of valuable genetic lines of founder/wild-caught individuals indefinitely, thereby permitting sustainable production of high-quality offspring over time (Cloud and Patton, 2009; Gwo, 2009; Cabrita et al., 2010). Moreover, maintaining a sperm bank of high-quality males is more economical and efficient than maintaining significant numbers of male broodstock. Given these advantages, it is not surprising that sperm cryopreservation protocols have been established in some decapod crustaceans, primarily in marine shrimp (Table 2.2).

Figure 2.1. Traditional and advanced predictive biomarkers of sperm quality and conditions that may contribute to improved male reproductive performance.

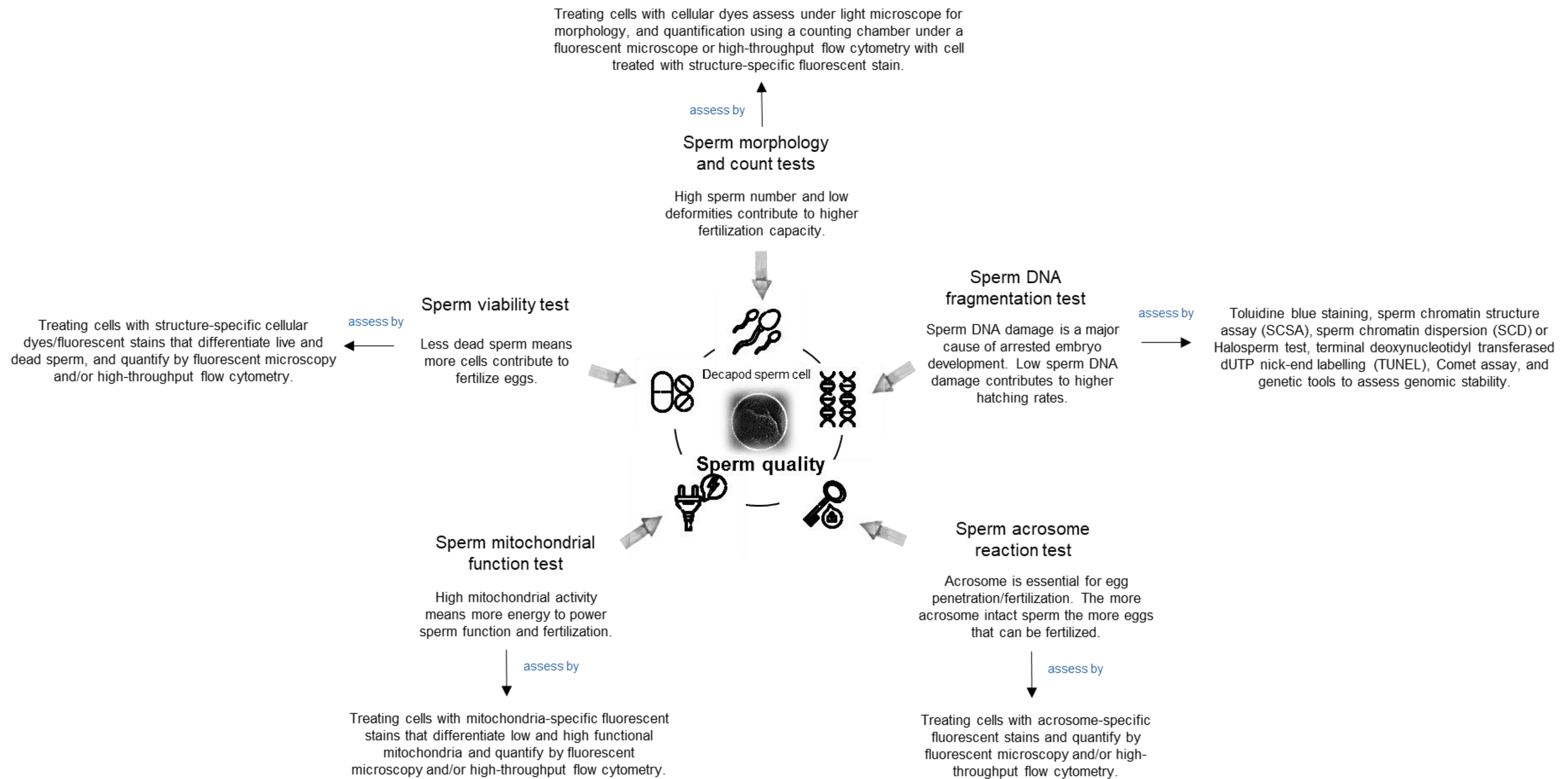


Table 2.2. Room temperature and chilled storage, long-term cryopreservation and vitrification of spermatophore and spermatozoa in decapod crustaceans.

Species	Semen collection method	Preserved tissue/cells	Holding solution/ cryoprotectant/ cooling vessel	Equilibration time/dilution ratio	Freezing rate & duration	Thawing conditions	Evaluation technique	Results	Reference
Freshwater crab									
Eriocheir sinensis	Manual extrusion	Spermatophore	Precooled (4 °C) Ca^{2+} - free artificial seawater + 5%DMSO & 10% glycerol in cryovials	Not determined	-1 ºC min^{-1} from room temp to -80 ºC; Plunged directly into -196 ºC	37 °C for 3 min	Light microscopy	AR in spermatozoa is promoted by cryopreservation	(Kang et al., 2009)
Freshwater prawn									
Macrobrachium rosenbergii	Manual removal from female's sternum	Spermatophore	Artificial seawater + 42 - 50% Ringer's solution in 50 ml vials	Not determined	Room temperature (20 – 25 ºC) for 17 h & 2 ºC for 4 d	Not mentioned	Artificial fertilisation (attached to female sternum using α-cyanoacrylate glue), fertilisation & hatching rates	Room temperature incubation resulted to 100% fertilisation & hatching success rates basing on the number of spermatophores fertilising eggs successfully (n=2). Chilled incubation of spermatophores resulted to 72.7 % fertilisation & hatching success rates basing on the number of spermatophores fertilising eggs successfully (n=11).	(Chow, 1982)
Macrobrachium rosenbergii	Manual removal from female's sternum	Spermatophore	Artificial seawater + 42 - 50% Ringer's solution in 50 ml vials	Room temp for 30 min; dilution ratio not mentioned	10 min in LN_2 vapour then -196 ºC for 20 d	30 ºC; time not mentioned	Artificial fertilisation, fertilisation & hatching rates	100% females had fertilised eggs, all of which hatched	(Chow et al., 1985)
Macrobrachium rosenbergii	Electroejaculation	Spermatophore	20% Ethylene glycol added dropwise to a 2-ml cryovial with a spermatophore.	Room temp for 15 min; dilution ratio not mentioned.	For -20ºC cryopreservation: immediate storage at -20ºC. For -196 ºC cryopreservation: equilibration at room temp for 15	30 ºC for 5 min.	Sperm viability via Trypan blue staining & fertilisation; light microscopy	10 or 20% glycerol or ethylene glycol at -20 ºC can store spermatophore for 10 days (80 - 90% sperm viability). For longer cryopreservation (150 days), 20% ethylene	(Akarasanon et al., 2004)

Species	Semen collection method	Preserved tissue/cells	Holding solution/ cryoprotectant/ cooling vessel	Equilibration time/dilution ratio	Freezing rate & duration	Thawing conditions	Evaluation technique	Results	Reference
					min then cooled to -70 ºC at -1.5 to -2.5 ºC min^{-1} in a cooling container filled with 95% ethanol. Dry-ice cubes (1 - 2 cm^3) were slowly dropped in the 95% ethanol. At -70 ºC, vials were exposed to LN_2 vapour (-110 ºC to -130 ºC) for 1-2 min before being plunged directly into LN_2 for 350 d.			glycol at -196 ºC is suitable (80 - 90% sperm viability) with fertilisation rates of > 60%.	
Macrobrachium rosenbergii	Electroejaculation	Sperm	Sterile-filtered pond water + 10% DMSO + 10% propylene glycol	Room temp for 30 min; dilution ratio not mentioned	-1.5 ºC min^{-1} cooling rate between 27 ºC & -39 ºC then stored in LN_2 for 90 d.	35 ºC for 1 min	Eosin–nigrosin staining, light, electron & phase contrast microscopy	Cryopreserved spermatophores: 50.4 ± 1.9% sperm viability. Cryopreserved sperm: 28.3 ± 2.2% acrosome reactivity compared to 85.3 ± 2.5% in fresh spermatophores.	(Valentina-Claudet et al., 2016)
Macrobrachium acanthurus	Electroejaculation	Spermatophore	Distilled water + 10% and 20% glycerol or 10% methanol in 2 ml plastic microtubes	25 ºC for 10 min; dilution ration not mentioned	-2 °C min^{-1}; equilibration time not mentioned	30 °C for 4 min	Eosin-nigrosin staining, light microscopy	Cold storage for up to 3 d: 35.3% sperm viability & 60 – 73% sperm viability using the cryoprotectants. Cooling rate at -2 °C min-1 resulted to 21.8% sperm viability.	(Costa et al., 2017)
				Marine crab					
Scylla serrata	Post-mortem dissection	Spermatophore, seminal plasma	Phosphate buffer (25 ml of 0.4 M NaCl/0.1 M glycine, 4 ml of 0.028 M NaH_2PO_4/0.072 M Na_2HPO_4 + cryoprotectants glycerol & DMSO + trehalose	4 ºC for 16 h; 1 seminal plasma:4 diluent	-79 ºC & -196 ºC for 30 d; straws were exposed to LN_2 vapour for 1 h then immersed & stored at -196 ºC in LN_2. For -79 ºC, straws were exposed to CO_2 for	Room temp; thawing duration not mentioned	Eosin-nigrosin staining for sperm viability	Sperm viability at -79 ºC: glycerol = 93.2 ± 1.0% DMSO-trehalose = 93.0 ± 1.2% Sperm viability at -196 ºC: glycerol = 95.3 ± 1.4%	(Jeyalectumie and Subramoniam, 1989)

Species	Semen collection method	Preserved tissue/cells	Holding solution/ cryoprotectant/ cooling vessel	Equilibration time/dilution ratio	Freezing rate & duration	Thawing conditions	Evaluation technique	Results	Reference
			combination) in 0.5 ml semen straws		1 h, and placed directly to dry ice.			DMSO-trehalose = 94.2 ± 0.0%	
Scylla serrata	Post-mortem dissection	Spermatophore, sperm	Ca^{2+} - filtered artificial seawater (FASW) + dropwise addition of glycerol (to 12.5%) in 0.5 ml straws sealed by polyvinyl alcohol	15 ºC for 10 min; dilution ratio not mentioned	Programmed controlled-rate freezing at -5 °C min^{-1} from different initial physiological temperatures (30 °C , 23 °C , & 15 °C) to various subzero temperatures (-30 °C to -50 °C) & subsequent storage at -196 °C with cryoprotectants	55°C for 10 – 15 sec	Artificial induction of acrosome reaction; hypo/hyperosmotic sensitivity tests for membrane integrity; trypan blue & eosin-nigrosin staining; light microscopy	DMSO, ethylene glycol & glycerol offered cryoprotection at 5% to 12.5% v/v. Post-thaw sperm viability highest (52%) in 12.5% glycerol at a cooling rate of -5 °C min^{-1}; 1% pronase releases sperm from spermatophores but can induce acrosome reaction thus mechanical shearing employed throughout experiments	(Bhavanishankar and Subramoniam, 1997)
Portunus trituberculatus	Post-mortem dissection	Sperm	Ca^{2+} - free saline + DMSO (percent not mentioned)	Not mentioned	Not mentioned	Not mentioned	Calcium ionophore A23187 treatment	Best preservation at 4 °C	(Shuai et al., 2007)
Charybdis japonica	Post-mortem dissection	Sperm	Not mentioned + 15% DMSO in cryovials	Not mentioned	Not mentioned	Not mentioned	Light microscopy	83.8% sperm viability after 24 h preservation & 73.8% after 1 year preservation in LN_2. 25 min pre-freezing incubation was appropriate for sperm cryopreservation.	(XingHong et al., 2010)
Charybdis japonica	Manual removal from female's spermatheca	Sperm	Sperm released from sperm mass using glass tissue grinder into Ca^{2+} - free saline + 15% DMSO in cryovials	Equilibration time not mentioned; 1 sperm mass : 2 buffer	Not mentioned	Not mentioned	Nα-benzol-DL-arginine-pnitroanilide (BAPNA) substrate method for acrosin activity, eosin B-staining, microscopy, SDS-PAGE	3 d cryopreservation at -196 ºC with 80.9 ± 1.0% sperm viability & 83.6 ± 1.7 μIU × 10^{-6} acrosin activity.	(Xu et al., 2014)
Scylla tranquebarica	Post-mortem dissection	Sperm	Ca^{2+} - free saline + 10% glycine in microcentrifuge tube	Room temp (25 ºC) for 60 min; 1 sperm suspension: 3 cryoprotectant	Not mentioned	Not mentioned	Eosin-nigrosin staining, light microscopy	Cryoprotectant: 10% glycine + extender yielded 84.8 ± 1.0% sperm viability	(Fatihah et al., 2018)

Species	Semen collection method	Preserved tissue/cells	Holding solution/ cryoprotectant/ cooling vessel	Equilibration time/dilution ratio	Freezing rate & duration	Thawing conditions	Evaluation technique	Results	Reference
Marine lobster									
Homarus americanus	Electroejaculation; Spermatophores cut in seawater & sperm mass extruded to form sperm suspension	Sperm suspension	Filtered seawater with 3 - 5 ml paraffin oil (Fisher, Saybolt viscosity 125/135) in Fisher culture tube	Not mentioned	4 – 7 ºC for 289 d	Not mentioned	Light & electron microscopy (EM)	Bacterial growth after > 289 d at 4 – 7 ºC. Sperm morphology using light microscopy. High-quality spermatozoa recovered after chilled storage < 289 d.	(Ishida et al., 1986)
Panulirus polyphagus	Post-mortem dissection	Sperm	Ca^{2+} - free saline + 10% glycine in 2 ml cryovials	Room temp (25 ºC) for 5 min; 1 spermatozoa:3 extenders	15 min at each of 25, 20, 16, 4, 2, -4, -20, -80, -150, and -196 ºC for 6 h	26 ºC for 30 sec	Eosin-nigrosin staining, light microscopy	Highest sperm viability in 10% glycine at 91.9 ± 2.0% (5 min at room temperature equilibration time), 91.3 ± 2.6% (6 h at -20 °C) & 75.9 ± 10.8% (6 h at -80 °C). Best thawing at 26 ºC for 30 sec with 76.1 ± 7.8% sperm viability	(Fatihah et al., 2016)
Marine shrimp									
Litopenaeus vannamei	Post-mortem dissection; Manual extrusion	Vas deferens	Filtered seawater or Ca^{2+} - free saline without cryoprotectant in 5 ml plastic vessel	15 ºC for 36 h	Not mentioned	Not mentioned	Phase-contrast microscopy	Seawater or Ca^{2+} - free saline useful for preparation & storage of gonadal tissue for gross morphology up to 36 h at 15 ºC. Sperm viability after 36 h at 15 ºC was 65.2 ± 21.6% (seawater) & 51.0 ± 6.8% (Ca^{2+} - F saline). Sperm viability assessed morphologically; observing abnormal sperm.	(Bray and Lawrence, 1998)
Fenneropenaeus indicus	Electroejaculation	Sperm	Filtered seawater (30 ppt) + Cryoprotectants: a) 5% DMSO + 5% glycerol	5 min at room temp, equal volume of cryoprotectant	Room temp to -35 ºC at -1 °C min^{-1}, then LN_2 vapour for 5 min before plunging into LN_2 for 60 d	20 °C; thawing duration not mentioned	Egg water-induced acrosome reaction; sperm viability	70 – 80% sperm viability after freezing at -35 °C & -196 °C	(Diwan and Joseph, 1998)

Species	Semen collection method	Preserved tissue/cells	Holding solution/ cryoprotectant/ cooling vessel	Equilibration time/dilution ratio	Freezing rate & duration	Thawing conditions	Evaluation technique	Results	Reference
			b) 5% DMSO + 0.25 M trehalose in 5 ml cryovials						
Litopenaeus vannamei	Manual extrusion	Spermatophore, spermatic mass, sperm suspension	Sterile sea water (SSW; 35 ppt) + 10% methanol or glycerol or 5% ethylene glycol in 0.5 ml French straws sealed with Polyvinyl alcohol	60 min for spermatophore & spermatic mass; 15 min sperm suspension; 1 sperm mass:10 SSW	-0.5 ºC min^{-1} from room temperature to -32 ºC, then immersed in LN_2 for 3 d. Manual seeding done at -6 ºC.	25 ºC for 40 sec for spermatophores, 20 ºC for 10 sec suspended in 0.2 M sucrose + SSW for sperm cells	Light microscopy; flow cytometry (DNA staining with propidium iodine)	Sperm viability after 2 h *in vitro*: SSW + 10% glycerol = 86.2 ± 4.5% SSW + 10% methanol = 85.6 ± 3.6% SSW + 5% ethylene glycol = 88.8 ± 1.1% Untreated SSW (control) = 87.0 ± 6.0% Sperm viability after 3 d: 61.6% using 10% methanol as cryoprotectant	(Lezcano et al., 2004)
Penaeus monodon	Manual extrusion	Spermatophore	Mineral oil as extender with 0.1% penicillin-streptomycin without cryoprotectant in closed Eppendorf tubes (opened for 10 min every 7 d for oxygen transfer)	Not mentioned	2 – 4 ºC for 8 d	Not mentioned	Eosin–nigrosin staining; light microscopy	58.3 ± 2.9% viable sperm in mineral oil with 0.1% penicillin-streptomycin. Fertilisation rate: 88.3 ± 0.9% Hatching rate: 87.6 ± 1.2%	(Nimrat et al., 2005)
Penaeus monodon	Manual extrusion	Spermatophore	Ca^{2+} - free 0.09% saline + 5% DMSO in cryovials	Spermatophore first in Ca^{2+} - free saline for 5 min, then transferred to cryoprotectant solution for 30 min	Two-step freezing rate used: firstly -15 ºC min^{-1} from 25 to -10 ºC, then -2 ºC min^{-1} from -10 to -80 ºC using a controlled rate freezer. Thereafter cryovials plunged in LN_2 for 48 h.	30 ºC for 2 min	Tissue weights; fertilisation; light microscopy	Frozen-thawed spermatophore has 79.7 ± 0.4% sperm viability with 79.9 ± 3.7% fertilisation rate & 87.8 ± 0.4% hatching rate	(Bart et al., 2006)
Litopenaeus vannamei	Manual extrusion	Spermatophore	Mineral oil with 0.1% penicillin-streptomycin without cryoprotectant in sterile Eppendorf tubes	Not mentioned	2 – 4 ºC for 35 d	Not mentioned	Eosin–nigrosin staining; light microscopy	Sperm viability (69.5 ± 3.9%) significantly higher ($P < 0.05$) among spermatophores preserved in mineral oil with 0.1% antibiotic compared with those preserved in	(Nimrat et al., 2006)

Species	Semen collection method	Preserved tissue/cells	Holding solution/ cryoprotectant/ cooling vessel	Equilibration time/dilution ratio	Freezing rate & duration	Thawing conditions	Evaluation technique	Results	Reference
								mineral oil only (57.7 ± 3.4%)	
Penaeus monodon	Manual extrusion	Spermatophore	Ca^{2+} - free saline + 5% DMSO in cryovials	30 min at 25 ºC; dilution ratio not mentioned	One-step cooling rate at -2 ºC min^{-1} between 25 & -80 ºC then storage in LN_2 for 60 d	30 ºC for 2 min	Eosin-nigrosin staining; light microscopy; fertilisation rate	Spermatophores cryopreserved for < 60 – 62 d: sperm viability: 87.3 ± 4.1%, fertilisation rate: 71.6 – 72.2%, hatching rate: 63.6 – 64.1% Long-term storage of spermatophores: sperm viability: 53.3 ± 4.3% (after 90 d), 46.7 ± 4.2% (120 d), < 40% (210 d) One-step cooling rate at -2 ºC min^{-1} resulted in 93.3 ± 2.7% sperm viability	(Vuthiphandchai et al., 2007)
Penaeus monodon	Manual extrusion	Spermatophore	Ca^{2+} - free saline + 5% DMSO in cryovials	30 min; dilution ratio not mentioned	One-step cooling rate at -2 ºC min^{-1} between 25 & -80 ºC then storage in LN_2 for 210 d	37 ºC for 2 min with cryovials wiped with 70% EtOH	Bacterial assays over time	Cryopreservation of spermatophores eliminated pathogenic bacteria during long-term storage in LN_2	(Nimrat et al., 2008)
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+} - free saline + 5% DMSO in cryovials	25 ºC for 30 min	-2 ºC min-1 from room temperature to -80 oC, maintained for 2 min, then plunged in LN_2 for 70 d	30 ºC for 2 min	Flow cytometry; eosin-nigrosin staining; light microscopy	Highest sperm viability of 34.4 ± 3.4% using this protocol followed by 33.3 ± 3.9% at -1 ºC min^{-1} freezing rate. Long-term cryopreservation: 30 min equilibration at 25 ºC in 5% DMSO yielded 49.5 ± 8.3% sperm viability in equilibrated sample, 44.3 ± 6.6% viability in samples frozen 1d, & 33.0 - 37.0% viability after 10, 20, 30, 40, 50, 60 & 70 d.	(Chao et al., 2009)

Species	Semen collection method	Preserved tissue/cells	Holding solution/ cryoprotectant/ cooling vessel	Equilibration time/dilution ratio	Freezing rate & duration	Thawing conditions	Evaluation technique	Results	Reference
Penaeus merguiensis	Manual extrusion	Spermatophore	Ca^{2+} - free saline + 15% $MgCl_2$ in cryovials	25 ºC for 15 min; dilution ratio not mentioned	10 min at each of 25, 20, 16, 4, 2, -4, -20, -80, -150 ºC for 90 d	27 ºC for 2 min	Eosin-nigrosin staining; light microscopy; fertilisation rate	Spermatophore cryopreserved with good sperm viability (55.4 ± 0.3%), fertilisation (64.1 ± 2.1%) & hatching (62.5 ± 1.5%) rates. Equilibrium time in $MgCl_2$ yielded 88.2 ± 7.3% viable sperm.	(Memon et al., 2012)
Litopenaeus vannamei	Manual extrusion	Sperm	Hank's balanced salt solution + antibiotic/antimycotic without cryoprotectants in microtubes	23 ºC for 12 h; 1:4 dilution	23º C for 4 h	Not mentioned	Fertilisation rate	92.0% viable sperm using modified artificial extenders with 60.4% female successfully spawned	(Morales-Ueno et al., 2013)
Litopenaeus schmitti	Post-mortem dissection	Sperm mass	Ca^{2+} - free saline + 5 % glycerol in cryovials	10 min; dilution ratio not mentioned	Two-step freezing protocol: 25 ºC to -6 ºC at -2 ºC min^{-1}, then -6 ºC to -32 ºC at -0.5 ºC min^{-1}, then sperm mass immersed in LN_2 for 15 d.	20 ºC for 10 sec	Eosin-nigrosin staining & light microscopy	5% glycerol yielded 17.2 ± 0.8% viable sperm after 30 d. Two-step freezing protocol achieved 42.9 ± 0.6% viable sperm after 15 d in LN_2.	(Chaves et al., 2014)
Litopenaeus vannamei	Manual extrusion	Sperm	Sterile seawater; Activation: Freezing solution (5% egg yolk & 0.2 M sucrose) + 5% DMSO or ethylene glycol in cryovials	30 min; dilution ratio not mentioned	Not mentioned	25 ºC for 40 sec	Eosin-nigrosin staining & light microscopy, flow cytometry	Short-term storage yielded 20 – 40% sperm viability after 30 d in LN_2.	(Uberti et al., 2014)
Litopenaeus vannamei	Manual extrusion	Sperm	Ca^{2+} - free saline + 30% methanol + 0.4 M trehalose + 2% soy lecithin in microtubes	25 ºC for 10 min; dilution ratio not mentioned	Room temperature (25 °C) for 10 min then vitrified directly in LN_2	1 ml Ca^{2+} - F saline; duration not mentioned	Eosin–nigrosin staining, fluorescent microscopy	30% Methanol for 120 min yielded 79.5 ± 1.3% sperm viability. Trehalose ± soy lecithin is an effective extracellular cryoprotectant for vitrification yielding 88.0 ± 1.6% sperm viability after 120 d.	(Castelo-Branco et al., 2015)
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+} - free saline + 0.4 M trehalose + 2%	25 ºC for 10 min; dilution	Vitrified directly in LN_2 for 150 d	36 ºC for 1 min	Light & fluorescent microscopy	91.8 ± 3.0% sperm viability using cryoprotectant with	(Castelo-Branco et al., 2018)

Species	Semen collection method	Preserved tissue/cells	Holding solution/ cryoprotectant/ cooling vessel	Equilibration time/dilution ratio	Freezing rate & duration	Thawing conditions	Evaluation technique	Results	Reference
			soy lecithin in microtubes	ratio not mentioned				73.0 ± 2.6% fertilisation rate but 0.0% hatching rate in all treatments.	
Penaeus monodon	Electroejaculation	Sperm	Ca^{2+} - free saline + 5% DMSO in cryovials	Room temp for 30 min; dilution ratio not mentioned	25 °C to -10 °C at -15 °C min^{-1}, then -10 °C to -80 °C at -2 °C min^{-1}, after which cryovials plunged into LN_2 for 180 d	28 °C for 2 min	Comet assay, sperm chromatin dispersion test (SCDt)	(1) % SDF was strongly correlated between SCDt & two-tailed comet assays (Pearson $r = 0.989$; $P = 0.01$) (2) % SDF did not increase due to mechanical stress induced by vortexing ($P = 0.76$) (3) % SDF was higher in domesticated (6.8 ± 4.5%) than wild (3.3 ± 1.5%) male broodstock ($P < 0.001$).	(Feng et al., 2018a)
Fenneropenaeus indicus	Manual extrusion	Sperm	Ca^{2+} - free saline + 5% DMSO + 5% MeOH + 10% egg yolk as co-cryoprotectant & 0.25 M trehalose in cryovials	Room temp for 30 min; dilution ratio not mentioned	-0.5 °C min^{-1} between 4 °C & -80 °C, hold for 5 min before storage in LN_2 for 45 d	30 °C for 1 min	Eosin-nigrosin staining; light microscopy; HOST & DNA integrity analyses; fluorescent microscopy	Sperm viability was 83.8 ± 2.5% after one-step slow freezing of spermatophores in 5% DMSO + 5% MeOH. Sperm quality was great at a freezing rate of -0.5 °C min^{-1} with 53.9 ± 4.9% sperm viability, 45.6 ± 4.2% HOST & 58.1 ± 1.7% DNA integrity.	(Selvakumar et al., 2018)

2.4.1. Cryoprotectants

Cryopreservation employs low temperatures to stop biochemical reactions and preserve living cells and tissues structurally intact. However, if not performed properly, freezing can damage cells via two distinct processes: ice crystal formation and concentrated solute effects (Bhattacharya, 2018). Freezing injury is caused by ice crystals that mechanically pierce or tear apart the cells and intracellular structures (Gao and Critser, 2000; Pegg, 2007). Solute effects, which include chemical and osmotic gradients created by concentrated salts in the residual unfrozen liquid between ice crystals, cause cell injury through dehydration. Preferential freezing of water over solutes results in concentration of solutes/salts (a hypertonic state) inside the cell. This hyperosmotic stress can lead to shrinkage and, eventually, cell death (Mazur et al., 1972; Gao and Critser, 2000).

Cryoprotectants (antifreeze agents) are water-soluble chemicals that decrease the melting point of water and are commonly used to avoid cell injury caused by freezing (Gao and Critser, 2000; Pegg, 2007). The absence of cryoprotectants generally results in the freezing of the entire water content inside the cell during cryopreservation, causing cell damage (Wowk, 2007; Bhattacharya, 2018). In order for a cryoprotectant to be effective and biologically compatible with living cells or tissues, it must possess the following properties: i) highly soluble in water even at low temperature; ii) capable of freely penetrating the cell membrane (except for non-penetrating cryoprotectants); and iii) little or no toxicity at concentrations needed for cryopreservation (Wowk, 2007; Elliott et al., 2017). Common cryoprotective agents include ethylene glycol, dimethylsulfoxide (DMSO), glycerol and propylene glycol, and typically have a working concentration of 5 – 15 % in carrier solutions (Pegg, 2007; Wowk, 2007; Sieme et al., 2016; Elliott et al., 2017; Bhattacharya, 2018).

Cryoprotectants can be cell-permeating or non-permeating (Gao and Critser, 2000; Wowk, 2007). Permeating cryoprotectants are typical of small molecular weight (< 100 Daltons) that facilitates easier penetration of cell membranes, making them osmotically inactive since they disperse equally in both extra- and intracellular spaces. This helps minimise the effect of excessive dehydration and ice crystal formation in cells during the freezing process (Gao and Critser, 2000; Wowk, 2007). Permeating cryoprotectants include DMSO, glycerol, ethylene glycol, methyl-formamide and dimethyl-formamide (Gao and Critser, 2000; Wowk, 2007). By contrast, non-permeating cryoprotectants, such as polyethylene glycol and polyvinylpyrrolidone, have larger molecular weights that prevent them from passing through the cell membrane (Gao and Critser, 2000; Wowk, 2007). These polymers are added to freezing solutions to inhibit ice crystal formation. At the same concentration, non-permeating cryoprotectants are generally less toxic than permeating ones (Wowk, 2007; Sieme et al., 2016; Elliott et al., 2017; Bhattacharya, 2018).

The use of cryoprotectants to freeze crustacean spermatozoa has been reported in several studies (Table 2.2). To date, ethylene glycol, dimethylsulfoxide (DMSO), glycerol, glycine, $MgCl_2$, methanol, soy lecithin and trehalose have been the main cryoprotectants used either alone or in combination with concentrations ranging from 5 – 20 % (Gao and Critser, 2000; Castelo-Branco et al., 2015; Castelo-Branco et al., 2018). These cryoprotectants were mixed with carrier solutions such as filtered fresh or seawater, Ringer’s, phosphate-buffered and Ca^{2+}-free saline. Spermatophores or spermatozoa usually were equilibrated with cryoprotectants for 5 – 60 min, at a ratio of 1:2, 1:3 or 1:4 spermatic mass to the freezing solution, depending on species (see Table 2.2).

2.4.2. Room temperature and chilled storage of crustacean spermatozoa

The spermatophore, spermatic mass, or spermatozoa of crustaceans have been held at room temperature (20 – 25 ºC), chilled (2 – 4 ºC) or cryopreserved in liquid nitrogen (-196 ºC; Chow, 1982; Morales-Ueno et al., 2013; Costa et al., 2017). For short-term storage of decapod spermatozoa, most studies reported good sperm viability in the absence of cryoprotectant. Short-term storage (4 – 17 h) of spermatophores at room temperature in Ringer's or Ca^{2+}- free saline without cryoprotectant maintained greater than 50 % sperm viability, fertilisation and hatching rates in both freshwater prawn *(Macrobrachium rosenbergii*; Chow, 1982) or Pacific white shrimp (*L. vannamei*), respectively (Morales-Ueno et al., 2013). Spermatophores from black tiger prawns and Pacific white prawns kept at 2 – 4 ºC in mineral oil with 0.1 % penicillin-streptomycin but no cryoprotectant exhibited 60 % sperm viability and 80 % fertilisation and hatching rates after 35 – 42 days (Nimrat et al., 2005; Nimrat et al., 2006). Moreover, clawed lobster (*Homarus americanus*) spermatophores preserved for up to 289 days in paraffin oil without cryoprotectant at 4 – 7 ºC maintained normal morphology as determined by phase contrast and electron microscopy (Ishida et al., 1986). However, storage for 3 days or longer at 2 ºC in distilled water containing 10 % glycerol led to low viability (35%) in giant freshwater prawn spermatozoa, which was attributed to the toxicity of the cryoprotectant (Costa et al., 2017). These reports suggest that cryoprotectants may be inappropriate for short-term storage of crustacean sperm above 0 ºC due to their toxic effect on metabolically active cells (Best, 2015).

2.4.3. Long-term cryopreservation

Using liquid nitrogen, spermatophores and spermatozoa of crustaceans have been reported to be preserved for between 90 – 180 days, depending on the species and cryoprotectant employed (Akarasanon et al., 2004; Memon et al., 2012; Valentina-Claudet et al., 2016; Feng et al., 2018a). Unlike vertebrate spermatozoa, sperm viability across several species of crustaceans appears to decline significantly beyond 180 days of storage (Gao and Critser, 2000; Vuthiphandchai et al., 2007). For crustaceans, the rate of cooling spermatozoa prior to freezing varies depending on species and generally ranges from -5 to -2.5 ºC min^{-1}, typically resulting in greater than 50 % viable spermatozoa (see Table 2.2). In most cases, frozen spermatophores or spermatozoa can be thawed at 20 – 30 ºC for 30 sec to 5 min depending on the species (see Table 2.2). In giant freshwater prawns, the best cooling rates for spermatozoa reportedly range from -1.5 to -2.5 ºC min^{-1} using either 10 % DMSO, 10 % propylene glycol (Valentina-Claudet et al., 2016), 10 % glycerol or 20 % ethylene glycol as cryoprotectants (Akarasanon et al., 2004). Their spermatozoa were subsequently best thawed in a 30 – 35 ºC water bath for 1 – 5 min (Chow et al., 1985; Akarasanon et al., 2004; Valentina-Claudet et al., 2016). These procedures resulted in the cryopreservation of spermatozoa at -196 ºC for 90 – 150 days with more than 50 % sperm viability (Akarasanon et al., 2004; Valentina-Claudet et al., 2016). The most frequently used cryoprotectant for Penaeid shrimp sperm cryopreservation is 5 % DMSO using either one- or two-step freezing rates. One-step freezing normally uses a rate of -2 ºC min^{-1} between 25 and -80 ºC before storing in liquid nitrogen (LN_2; Vuthiphandchai et al., 2007; Nimrat et al., 2008). Two-step freezing involves lowering of temperature at -15 ºC min^{-1} from 25 to -10 ºC, then at -2 ºC min^{-1} from -10 to -80 ºC. Thereafter, cryovials are plunged in LN_2 at -196 ºC (Bart et al., 2006; Feng et al., 2018a). Frozen spermatozoa are thawed in a 27 – 30 ºC water bath for 2 min (Bart et al., 2006;

Vuthiphandchai et al., 2007; Nimrat et al., 2008; Memon et al., 2012; Feng et al., 2018a). Using these procedures, more than 50 % sperm viability can be achieved following 90 – 180 days of cryopreservation (Bart et al., 2006; Vuthiphandchai et al., 2007; Nimrat et al., 2008; Memon et al., 2012; Feng et al., 2018a). In giant mud crabs (*Scylla serrata*), spermatozoa can be cryopreserved in LN_2 using 15 % glycerol or 5 % DMSO and 0.25 M trehalose as cryoprotectants for up to 30 days with more than 90 % sperm viability (Jeyalectumie and Subramoniam, 1989). In another study, giant mud crab spermatozoa exhibited 50 % viability after 8 h frozen in LN_2 at a cooling rate of -5 ºC min^{-1} using 12.5 % glycerol as the cryoprotectant and thawing in a 55 °C water bath for 10 – 15 sec (Bhavanishankar and Subramoniam, 1997). In mud spiny lobster (*Panulirus polyphagus*), spermatozoa exhibited up to 80 % viability after 24 h cryopreservation using 10 % glycine as cryoprotectant and a freezing rate of 15 min at each of 8 graded temperatures (25, 20, 16, 4, 2, -4, -20, -80, and -150 °C), followed by immediate storage in LN_2, and thawing at 26 ºC for 30 sec (Fatihah et al., 2016).

Post-thaw sperm quality is usually evaluated by measuring their fertilisation and hatching rates in crustaceans (Chow et al., 1985; Akarasanon et al., 2004; Bart et al., 2006; Vuthiphandchai et al., 2007). In giant freshwater prawns, spermatophores equilibrated in freshwater for 15 – 30 min after thawing was attached to the female's sternum using α-cyanoacrylate as a glue, resulting in successful fertilisation and hatching (though the authors did not report the rates (Chow et al., 1985). In black tiger prawns, two studies reported 70 – 89 % fertilisation rates and 63 – 88 % hatching rates after artificial fertilisation using cryopreserved spermatophores (Bart et al., 2006; Vuthiphandchai et al., 2007). In summary, both short- and long-term storage of sperm at room temperature by chilling or cryopreservation in liquid nitrogen has been reported and appears relatively successful in

different taxonomic groups of crustaceans, including freshwater prawn and marine shrimp, crabs, and marine lobsters (Chow, 1982; Chow et al., 1985; Ishida et al., 1986; Akarasanon et al., 2004; Nimrat et al., 2005; Bart et al., 2006; Nimrat et al., 2006; Vuthiphandchai et al., 2007; Nimrat et al., 2008; Memon et al., 2012; Fatihah et al., 2016; Valentina-Claudet et al., 2016; Fatihah et al., 2018; Feng et al., 2018a). Nevertheless, further work in this area to optimise freezing protocols for other decapod crustaceans is required.

2.4.4. Sperm vitrification

An alternative to conventional sperm cryopreservation is the rapid freezing of spermatozoa by a process known as vitrification. This process, through an extreme increase in viscosity during freezing, causes the solidification of liquid into an amorphous or glassy state at low temperatures to avoid inducing ice formation and crystallisation (Pegg, 2007; Wowk, 2007; Li et al., 2019). If the freezing of viscous solution occurs rapidly, the supercooled liquid retains its physical liquid properties until it reaches its glass-state transition temperature. Rapid freezing below this temperature maintains the disorderly organisation of the solution’s molecules, but its physical properties remain rigidly solid. In this state, molecules are locked in place as if the liquid were frozen in time, resulting in a “solid liquid” known as “glass” (Wowk, 2010).

For the vitrification process, cells need to be exposed to a high concentration of cryoprotectant solution early. Rapid cooling allows the entire volume of a cell to change to a glassy solid state (vitrifying), devoid of freezing (Pegg, 2007; Wowk, 2007; Elliott et al., 2017; Magnotti et al., 2018; Li et al., 2019). In decapods, whiteleg shrimp spermatozoa were equilibrated for 10 min at room temperature (25 ºC) in several different cryoprotectant

solutions: (i) Ca^{2+}- free saline + 0.4 M trehalose (base solution), (ii) base solution + 30 % methanol (MeOH), (iii) base solution + 30 % MeOH + 1 % soy lecithin, (iv) base solution + 30 % MeOH + 2 % soy lecithin, (v) base solution + 1 % soy lecithin and (vi) base solution + 2 % soy lecithin, before plunging them directly into LN_2 (-196 ºC; Castelo-Branco et al., 2015). Results showed that trehalose alone (base solution; 90.1 ± 2.4 % sperm viability) or trehalose + 2 % lecithin (91.1 ± 3.9 % sperm viability) were effective extracellular cryoprotectants for vitrification after 120 days of storage in LN_2 (Castelo-Branco et al., 2015). In a subsequent study, whiteleg shrimp spermatozoa exceeded 90 % viability after 150 days in LN_2 using 0.4 M trehalose + 2 % lecithin as the cryoprotectants (Castelo-Branco et al., 2018). However, artificial fertilisation with thawed spermatozoa preserved by this method resulted in extremely poor to zero hatching rates, suggesting that the vitrification process may have induced high rates of sperm DNA damage (but this remains to be determined - emphasising the need to develop sperm DNA damage assays for crustaceans). Clearly, the application of conventional slow-freezing or vitrification techniques to the aquaculture industry would revolutionise the maintenance of male broodstock genetics in sperm banks and, by so doing, significantly reduce operational expenses associated with broodstock management. Coupled with the development of successful techniques to efficiently reinfuse these genetics back into broodstock females via procedures such as artificial fertilisation, such advanced reproductive technologies could provide better control of reproduction and selective breeding of valuable genetic lines of economically important decapod crustaceans.

2.5. Natural and artificial fertilisation in decapod crustaceans

Understanding the physiological changes to gametes during fertilisation in decapod crustaceans is essential for improving hatchery management, selective breeding, and other

reproductive strategies. Changes in sperm physiology and mating behaviour are particularly well documented in freshwater crayfish (Harlıoğlu and Farhadi, 2017; Yazicioglu et al., 2018; Farhadi and Harlıoğlu, 2019); therefore, the subsequent description will primarily focus on this group of decapod crustaceans. During mating, the thick muscle of the distal vas deferens contracts, and a segment of the sperm cord (a continuous cord containing spermatozoa surrounded by primary and secondary secretions) is squeezed through the gonopores and ejected by the *appendices masculinae*. The internal sperm cord is fragmented upon extrusion, releasing an individual spermatophore unit. Only one spermatophore is transferred to a female's sternum at a time and forms a sticky opalescent mass within 10 min post-extrusion. By 1 h post-extrusion, the spermatophore begins to harden and completely solidifies within 24 h. At this point, the female crayfish has already separated from the male, extends her pleon, folds her abdomen, and expands her uropods to create a temporary brood chamber. Eggs are visible within the chamber, and some are already attached to the pleopod setae, with all eggs firmly attached after 48 h. Hydration of the attached spermatophore occurs 24 – 48 h post-mating; confirmed by increased swelling and coiling of the sperm cord, thereby increasing its size (López Greco and Lo Nostro, 2008). After 72 h, the spermatophore softens and between 72 – 96 h post-mating, many sections of the sperm cord begin to coalesce. Fissures are observed in the matrix of the secondary spermatophore layer from 48 h, which increase further after 72 – 120 h post-mating. Ultimately, the spermatophore disintegrates completely between 96 – 120 h (López Greco and Lo Nostro, 2008). After mating, the female releases a secretion from her glair glands that dissolves the wall of the spermatophore, allowing her to smear spermatozoa across her ventral abdomen before egg release. Glair secretions are sufficient to firmly hold spermatozoa and prevent them from being washed away until fertilisation is complete (Niksirat et al., 2014b). The female regulates the timing of sperm release by breaking open the primary spermatophore layer and

drawing spermatozoa into her brood chamber to facilitate fertilisation (Jones, 1995; López Greco and Lo Nostro, 2008). The spermatophore is completely ruptured after egg release and attachment to the pleopods, enabling the female to manipulate spermatozoa into her brood chamber to complete external fertilisation (López Greco and Lo Nostro, 2008). The female brood chamber is maintained to incubate fertilised eggs for 5 – 7 days post-oviposition. After this time, her abdomen is extended to gently ventilate the visibly fertilised eggs using her swimming legs (López Greco and Lo Nostro, 2008). The time of natural mating and egg-laying differs across decapod crustaceans depending on water temperature, but this can be controlled artificially by manipulating temperature and photoperiod (Jones, 1995; Parnes and Sagi, 2002; Reynolds, 2002).

2.5.1. Spermatophore structure and function

Across decapod crustaceans, the spermatophore varies morphologically and can be classified into three general types. The first type forms a small round or ellipsoid shape usually found in brachyuran crabs (e.g., blue-swimmer crabs and giant mud crabs). This type of spermatophore is suspended in seminal fluid and deposited in the female spermatheca during true copulation. In brachyuran crabs, spermatophores degenerate after copulation and lose their protective function, only serving to keep spermatozoa together during transfer to the female prior to internal fertilisation (Subramoniam, 2017c). The second type is the pedunculated spermatophore, usually found in anomuran crabs (e.g., hermit crabs, mole crabs and sand crabs). This type of spermatophore forms a stalk or peduncle that is fixed onto the ventral sternum of the female during copulation, where it remains for an extended period until the female mechanically manipulates it for external fertilisation (Subramoniam, 2017c). The third type of spermatophore, produced by most macrurans (e.g., crayfish and lobsters),

contains spermatozoa enclosed within a sperm cord surrounded by one or more layers of protective gelatinous matrix. This type of spermatophore is also produced by penaeid shrimps but with accessory structures such as attachment wings (Subramoniam, 2017c). Generally, this type of spermatophore is deposited on the ventral sternum of female crayfish and lobsters or inserted in the thelycum (sex organ) of female penaeid shrimp as well as homarid and nephrosis lobsters (Subramoniam, 2017c). In freshwater crayfish, spermatophores harden upon exposure to water. This hardening protects spermatozoa from physical damage brought on by environmental stressors, thereby enhancing sperm viability (Dudenhausen and Talbot, 1983; López Greco and Lo Nostro, 2008; Niksirat et al., 2014b; Farhadi and Harlıoğlu, 2019). Hardening of the secondary spermatophore layer is caused by enzymatic reactions associated with calcification of chitin complexes. Moreover, highly acidic mucopolysaccharides (containing chondroitin sulphate and hyaluronic acid) contribute to the calcification and antimicrobial activity of the spermatophore. Chondroitin sulphate aids the desiccation of spermatophores by providing elasticity and resistance to compression (Subramoniam, 1993; Niksirat et al., 2014b; Subramoniam, 2017c). Calcium-related proteins such as sarcoplasmic calcium-binding protein, crustacean calcium-binding protein 23, ryanodine receptor, and troponin C2 have been identified in the spermatophore of freshwater crayfish and are suspected to be responsible for calcification during hardening (Niksirat et al., 2014a; Niksirat et al., 2014b; Niksirat et al., 2015a; Niksirat and Kouba, 2016; Farhadi and Harlıoğlu, 2019).

2.5.2. Natural fertilisation

Spermatozoa encased by a spermatophore are unable to fertilise; only after chemical 'activation' do they acquire the capacity to fertilise; a process that is time-dependent (Fraser,

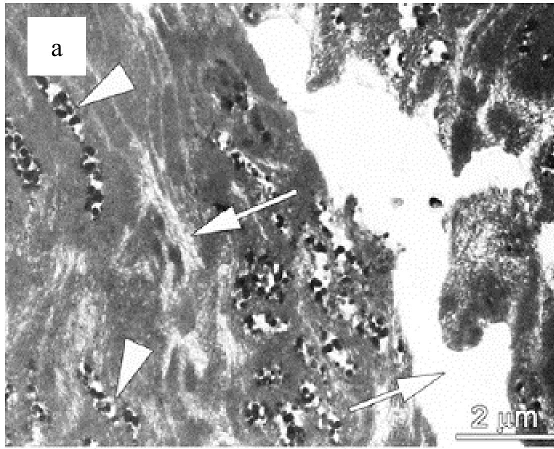
1995, 1998). These morphological and molecular changes are known as sperm capacitation (Niksirat et al., 2014b; Niksirat et al., 2015a; Niksirat et al., 2016; Farhadi and Harlıoğlu, 2019). Sperm capacitation in crustaceans occurs either inside seminal receptacles, such as the thelycum in Penaeid shrimp, or on the ventral portion of the female, such as in freshwater crayfish (Alfaro et al., 2003; Aungsuchawan et al., 2011; Subramoniam, 2017c; Farhadi and Harlıoğlu, 2019). During the process, both spermatophores and spermatozoa alter their morphology. In freshwater crayfish, for example, the release of glair gland secretions by the female to dissolve the hard wall of the spermatophore is known to initiate sperm capacitation prior to fertilisation (Niksirat et al., 2014b). Changes to the spermatozoon occur primarily in the extracellular capsule, plasma membrane and subacrosomal zone after the spermatophore is dissolved. During capacitation, the extracellular capsule swells, and space appears between the spermatozoa and the capsule. The anterior portion of the acrosome changes from a single- to a multi-layered structure, and the plasma membrane wrinkles, thus increasing the wrapping of the membrane around the acrosomal apical cap (Niksirat et al., 2014b; Farhadi and Harlıoğlu, 2019). After capacitation, the acrosome reaction involves morphological and molecular changes to the acrosomal vesicle and the discharge of subacrosomal and nuclear material into the egg (Talbot and Chanmanon, 1980; Niksirat et al., 2014b; Farhadi and Harlıoğlu, 2019). The subacrosomal zone detaches from the main acrosome, losing electron density, and membranous lamellae separate from the free spermatozoon. Electron-dense material that wraps the filaments in the innermost portion of the acrosome pre- and post-mating is released from the spermatozoon (Figure 2.2; Niksirat et al., 2014b). The discharge of subacrosomal contents is aided by its morphological alteration during the process. The subacrosomal zone is calcified, thus increasing its ability to puncture the egg membranes in order to transfer nuclear material into the egg successfully (Niksirat and Kouba, 2016; Farhadi and Harlıoğlu, 2019). Discharge of the inner nuclear material occurs simultaneously

with the acrosome filaments, forming a droplet or filament structure in the anterior portion of the spermatozoon (Niksirat et al., 2014a; Niksirat et al., 2014b; Farhadi and Harlıoğlu, 2019). After the discharge of the nuclear material, the extracellular capsule, membranous lamellae, and plasma membrane are eliminated. Nuclear material at this time is less condensed than in spermatozoa at earlier stages (Niksirat and Kouba, 2016; Farhadi and Harlıoğlu, 2019). Transfer of nuclear material inside the egg activates an instantaneous electrical block to protect the egg from polyspermy - fertilisation by multiple spermatozoa (Goudeau and Goudeau, 1989; Tsai and Talbot, 1993; Gould and Stephano, 2003; Niksirat et al., 2015b).

In other decapod crustaceans such as black tiger prawn (*P. monodon*) and pink prawn (*F. paulensis*) that have a closed thelycum, capacitated spermatozoa have a more electron-dense acrosome and subacrosomal zone and less condensed nucleus (Vanichviriyakit et al., 2004; Braga et al., 2014). Similar to sperm capacitation in mammals, changes in protein composition of the plasma membrane and increasing protein tyrosine phosphorylation have been observed (Visconti et al., 1995; Vanichviriyakit et al., 2004). After the acrosome reaction, the apical cap becomes less concave (Vanichviriyakit et al., 2004; Braga et al., 2014). In decapods with open thelycum, such as the whiteleg shrimp (*L. vannamei*), copulation is accomplished by attaching the male spermatophore onto the surface of the thelycum 4 – 6 h before spawning. After attachment, ultrastructural changes associated with sperm capacitation involve the formation of a filamentous meshwork between the sperm nucleus and hemispherical cap (Alfaro et al., 2007; Aungsuchawan et al., 2011). Most reports describing the acrosome reaction in shrimp spermatozoa are based on artificial induction by egg water *in vitro* and, as such, are not as specific as *in vivo* acrosome reaction reported in freshwater crayfish (Clark Jr and Griffin, 1988; Alfaro et al., 2007; Aungsuchawan et al., 2011). In black tiger shrimp, a mature spermatozoon consists of three major regions: anterior,

middle, and posterior. An acrosomal spike is visible on the anterior region, while the posterior region contains the nucleus and forms the main body of the spermatozoon. The subacrosomal materials occur in the middle region. During egg water-induced acrosome reaction *in vitro*, the acrosome loses its spike (termed depolymerisation) followed by acrosomal exocytosis of nuclear materials (Pongtippatee et al., 2007; Kruevaisayawan et al., 2008; Subramoniam, 2017b). Upon sperm contact with the vitelline egg envelope during natural fertilisation, the acrosomal spike also degenerates, and an electron-dense spherical mass is formed (Pongtippatee et al., 2007; Kruevaisayawan et al., 2008). The spherical mass draws the nuclear material into the egg's cytoplasm during sperm entry (Pongtippatee et al., 2007).

In crabs, acrosome reaction occurs simultaneously with sperm penetration into the oocyte membrane (Subramoniam, 2017b). Once the acrosome reaction is complete, a fertilisation cone is formed at the site of sperm-egg contact. Between the acrosome tubule and the fertilisation cone, bell-shaped corpuscles are observed that are believed to release a binding-like substance to stimulate sperm-egg membrane fusion (Goudeau and Jacqueline, 1982; Subramoniam, 2017b). Since sperm morphology varies across different species of decapod crustaceans, our general understanding of the process of natural fertilisation is still largely inadequate. In particular, the molecular mechanisms underlying sperm capacitation, acrosome reaction, and fertilisation of eggs require further research that will ultimately improve our ability to develop successful artificial fertilisation techniques.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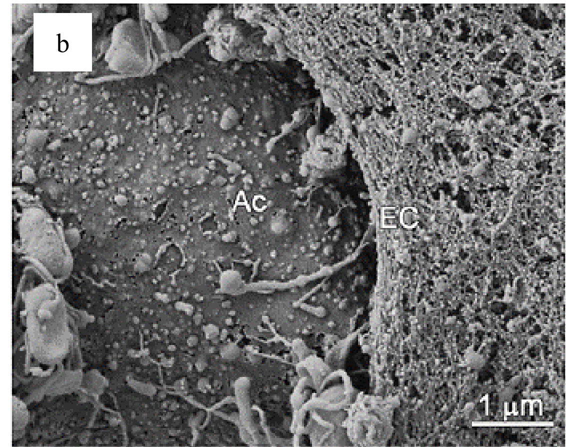
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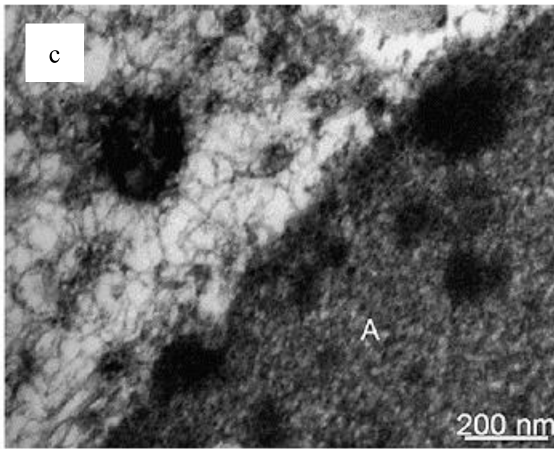
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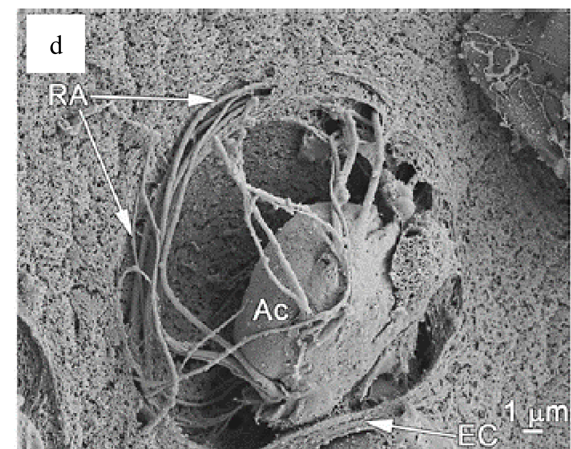
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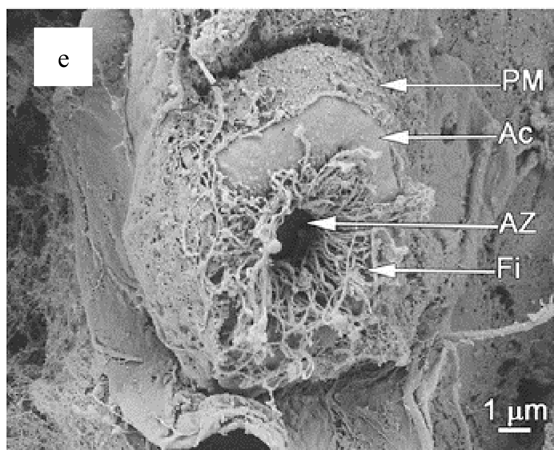
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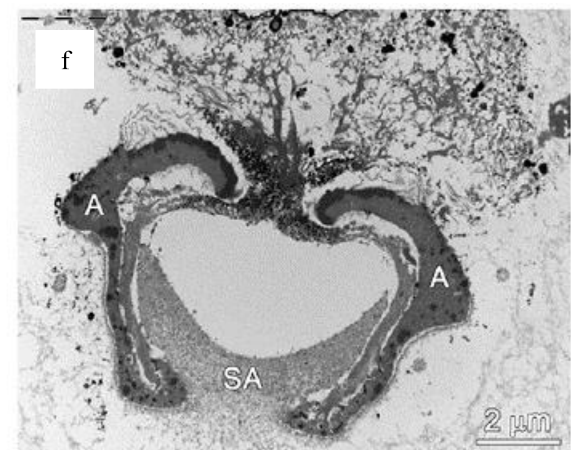
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Figure 2.2. Electron (transmission and scanning) micrographs of the spermatophore wall and released spermatozoa in narrow-clawed crayfish (*A. leptodactylus*). (a) dissolving wall of the spermatophore with distorted granules (arrowheads) and areas where female glair secretions have penetrated (arrows); (b) exposed anterior acrosome secreting its contents in numerous droplets; (c) sagittal close-up showing acrosomal secretion with electron-dense vesicles at the inner surface of the acrosome being discharged extracellularly (d) a spermatozoon released from the extracellular capsule with nuclear radial arms and acrosomal complex clearly visible; (e) discharge of filaments from the anterior portion of the acrosome causing a cavity; (f) sagittal section of a spermatozoon extruding filaments and electron-dense material from the apical zone of the acrosome leaving a withdrawn subacrosomal zone; (g) close-up of

electron-dense material being expelled via the apical zone; (h) cross-section of remaining acrosome layers and subacrosome zone after release of electron-dense materials; (i) boundary between subacrosome zone and nucleus (arrowheads). A, acrosome; Ac, acrosome complex; AM, acrosomal membrane; AZ, apical zone; EC, extracellular capsule; Fi, filaments; IL, inner acrosome layer; ML, middle acrosome layer; N, nucleus; PM, residual plasma membrane; RA, radial arms; SA, subacrosomal zone (Niksirat et. al., 2014).

2.5.3. Artificial fertilisation

In traditional aquaculture of economically important crustaceans, animals might only reach sexual maturity and reproduce in captivity when conditions are favourable such as the black tiger prawn. Moreover, males and females of some aquaculture species may not mature and reproduce simultaneously in captivity (Haldar et al., 2018). With the intensification of aquaculture, interest in controlling crustacean reproduction using assisted breeding techniques has gained great impetus (Cabrita et al., 2014; Beirão et al., 2019). With such techniques, gametes can be collected from superior broodstock with high-quality phenotypic traits and used for artificial fertilisation during a precisely controlled time window (Haldar et al., 2018), ensuring genetic selection for high-quality offspring can be maximised for commercial production.

Artificial fertilisation (AF) in crustaceans involves either the introduction of sperm extruded from the male into the female sex organ for internal fertilisers or adhesion of the spermatophore near the ventral gonopores of the female for external fertilisers (Haldar et al., 2018). AF is a means of reproductive control that allows fertilisation to occur anytime, which should dramatically speed up selective breeding programs while dramatically reducing the

cost (Haldar et al., 2018). Due to unique differences in reproductive physiology, fertilisation and hatching rates after AF varies in each species of decapod (Subramoniam, 2017b; Beirão et al., 2019). Although previous studies reported high spawning rates, they yielded few or no embryos (Chow et al., 1985; Farhadi et al., 2019a). Despite not yet being widely employed, the external nature of fertilisation in many decapods makes them ideal candidates for AF, which has been attempted and reported in a number of species. For example, for freshwater prawn AF, spermatophores were extruded by post-mortem dissection (Sandifer and Smith, 1979), manual extrusion (Sandifer and Lynn, 1980), electroejaculation (Sandifer et al., 1984; Harris and Sandifer, 1986), or direct removal from the female's sternum or seminal receptacles post-copulation (Chow, 1982; Chow et al., 1985). Generally, both fresh and cryopreserved spermatophores can be utilised for artificial fertilisation (Akarasanon et al., 2004; Beirão et al., 2019). Female freshwater prawns were fertilised artificially using a retainer tube with their ventral side facing the operator (Sandifer and Smith, 1979; Chow, 1982; Chow et al., 1985). AF efficiency was evaluated through sperm counts, fertilisation and hatching rates of eggs and was highly successful in laboratory experiments in freshwater prawn and penaeid shrimp (Akarasanon et al., 2004; Vuthiphandchai et al., 2007; Beirão et al., 2019). Using AF, 75 – 100 % of female freshwater prawns had fertilised eggs with 90 – 100 % hatching rates using either fresh or cryopreserved spermatophores (Chow, 1982; Chow et al., 1985). In one study, fertilising capacity was determined by evaluating the survival rate of developing embryos five days after spawning, as well as estimating the rate of effective spermatophores (spermatophores that yield normal hatching of fertilised embryos) after adhesion (Akarasanon et al., 2004). High embryo survival rates (75 – 90 %) were observed using cryopreserved spermatophores stored in LN_2 for 30 – 100 days (Akarasanon et al., 2004). Moreover, high male fecundity after AF appeared directly proportional to sperm concentration in a given spermatophore, demonstrating the direct link between sperm quality

and egg fertilisation and hatching rates. By contrast, in narrow-clawed freshwater crayfish, freshly electroejaculated spermatophores with 80 % sperm viability failed to fertilise eggs nor produce offspring, suggesting that electroejaculated spermatophores in this study may have contained significantly fewer spermatozoa to participate in fertilisation (Farhadi et al., 2019a). Furthermore, whiteleg shrimp spermatozoa with > 90 % viability after spermatophore vitrification achieved > 70 % fertilisation rates but had low to no hatching after artificial fertilisation (Castelo-Branco et al., 2018). The inability of these embryos to survive until hatching was thought to be caused by fertilisation with vitrification-induced DNA-damaged spermatozoa, but this claim remains to be validated (Castelo-Branco et al., 2018). These above preliminary studies offer hope for the commercial development of AF in selected decapods, while for others, greater understanding of the underlying mechanism and timing of gamete interaction during fertilisation is required before sperm cryopreservation and AF can be of benefit to their commercial aquaculture.

2.6. Conclusions

This review highlights several alternative biomarkers of male fertility, including plasma membrane integrity, mitochondrial function, acrosome reaction, and DNA fragmentation in spermatozoa, which are likely to be of value but are still in their infancy of application in decapod crustaceans. Functional measurement of these intracellular sperm organelles has been demonstrated as a reliable indicator of sperm fertilisation competence. The use of fluorescent cellular dyes coupled with high-throughput flow cytometry enables rapid and accurate analysis of large numbers of freshly stained spermatozoa per animal in a short period of time. Optimisation of these technologies at the species-specific level in decapod

crustaceans is clearly required; although standardisation of protocols at the genus level would accelerate their uptake and so should be given high priority.

Application of conventional sperm cryopreservation or vitrification techniques to crustacean aquaculture would revolutionise the maintenance of male broodstock genetics in Sperm Banks and, by so doing, significantly reduce operational expenses associated with broodstock management. Coupled with techniques to reinfuse these genetics back into broodstock females via procedures such as artificial fertilisation, these advanced reproductive technologies will provide better control of reproduction and selective breeding of valuable genetic lines of decapod crustaceans.

Advanced reproductive tools to assess male fertility can be further used as sensitive biomarkers to improve sperm handling procedures, broodstock husbandry and nutrition. Successful application of these advanced diagnostic tools to decapods could help identify the impact male broodstock with low sperm quality may have on poor offspring yield or survival, particularly in the case of low sperm quality caused by factors such as DNA damage that is undetectable by traditional methods, thereby allowing breeders to make informed decisions about which males to discard in their broodstock. Males identified with high-quality spermatozoa can be retained for natural breeding, or their spermatozoa can be used for downstream cryopreservation and artificial fertilisation. In turn, this improves overall reproductive performance, productivity and cost-efficient management of the hatchery. In addition, formulation of broodstock diet and adjustment of environmental conditions to optimise male reproductive performance can be achieved through evaluation of sperm quality with the application of advanced diagnostic tools specific for decapods. Production of decapod crustaceans can be substantially improved by careful selection of broodstock males

at each successive generation based on both physical (e.g., absence of body damage, appropriate size at sexual maturity, body pigmentation, complete appendages, disease resistance, growth and moulting rates) and reproductive (sperm quality) traits (Figure 2.3). These technologies can help breeders make better decisions about broodstock management leading to greater numbers of superior progeny, thus accelerating both commercial crustacean aquaculture production and stock enhancement programmes.

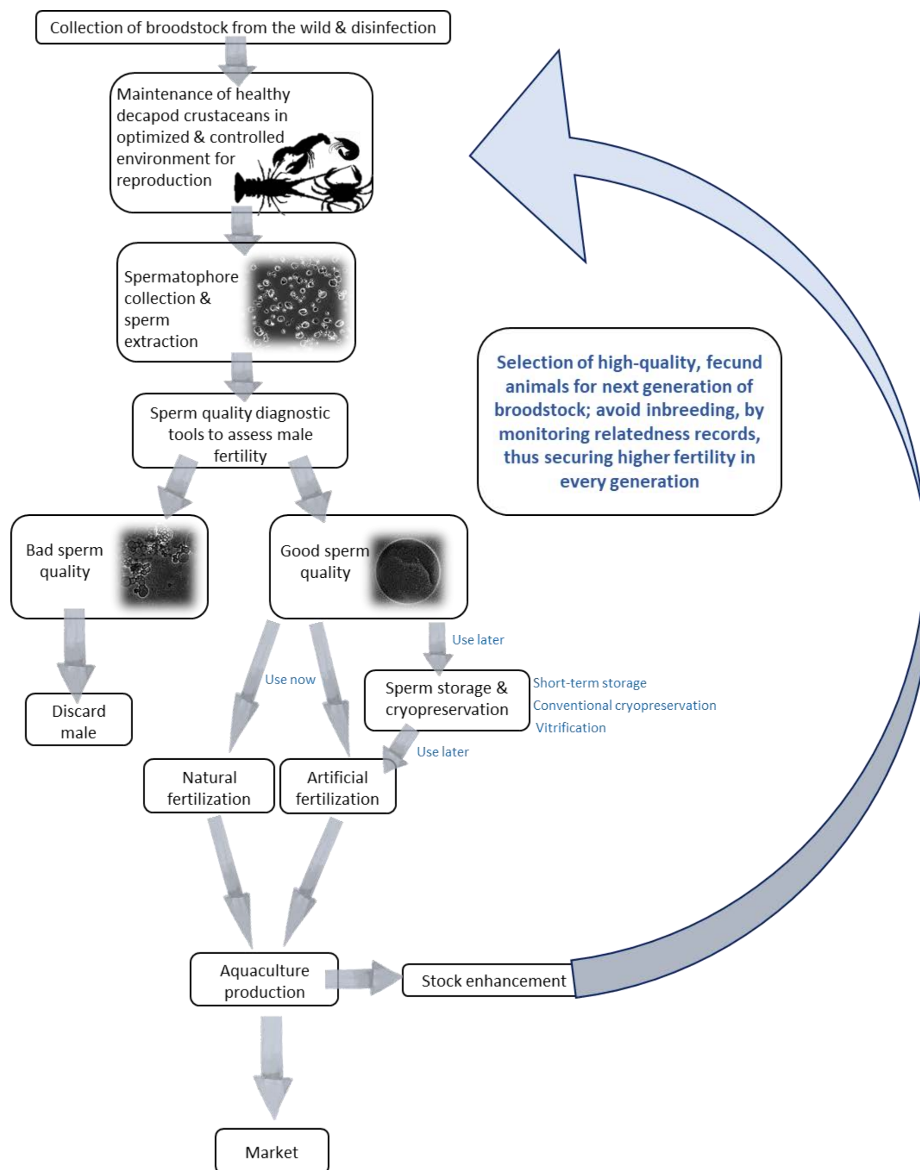

Figure 2.3. Application of advanced reproductive tools to improve sustainable production of economically important decapod crustaceans in aquaculture.

2.7. Acknowledgements

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2.8. References

Adams, S.L., Hessian, P.A., Mladenov, P.V., 2003. Flow cytometric evaluation of mitochondrial function and membrane integrity of marine invertebrate sperm. Invertebr. Reprod. Dev. 44(1), 45-51. https://doi.org/https://doi.org/10.1080/07924259.2003.9652552.

Agarwal, A., Qiu, E., Sharma, R., 2017. Laboratory assessment of oxidative stress in semen. Arab J. Urol. 16(1), 77-86. https://doi.org/https://doi.org/10.1016/j.aju.2017.11.008.

Ahmadi, A., Ng, S.-C., 1999. Fertilizing ability of DNA-damaged spermatozoa. J. Exp. Zool. 284(6), 696-704. https://doi.org/https://doi.org/10.1002/(SICI)1097-010X(19991101)284:6<696::AID-JEZ11>3.0.CO;2-E.

Aiken, D.E., Waddy, S.L., Moreland, K., Polar, S.M., 1984. Electrically induced ejaculation and artificial insemination of the american lobster *Homarus Americanus*. J. Crust. Biol. 4(4), 519-527. https://doi.org/https://doi.org/10.2307/1548065.

Aitken, R.J., Jones, K.T., Robertson, S.A., 2012. Reactive oxygen species and sperm function - In sickness and in health. J. Androl 33(6), 1096-1106. https://doi.org/https://doi.org/10.2164/jandrol.112.016535.

Aitken, R.J., Koopman, P., Lewis, S.E.M., 2004. Seeds of concern. Nature 432(7013), 48-52. https://doi.org/https://doi.org/10.1038/432048a.

Akarasanon, K., Damrongphol, P., Poolsanguan, W., 2004. Long-term cryopreservation of spermatophore of the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). Aquac. Res. 35(15), 1415-1420. https://doi.org/https://doi.org/10.1111/j.1365-2109.2004.01163.x.

Alfaro, J., Muñoz, N., Vargas, M., Komen, J., 2003. Induction of sperm activation in open and closed thelycum penaeoid shrimps. Aquaculture 216(1), 371-381. https://doi.org/https://doi.org/10.1016/S0044-8486(02)00514-8.

Alfaro, J., Ulate, K., Vargas, M., 2007. Sperm maturation and capacitation in the open thelycum shrimp *Litopenaeus* (Crustacea: Decapoda: Penaeoidea). Aquaculture 270(1), 436-442. https://doi.org/https://doi.org/10.1016/j.aquaculture.2007.05.011.

An, C.-G., Weng, X.-L., Xu, Y.-Z., Fan, Y.-J., Zhao, Y.-L., 2011. Histological and ultrastructural studies on the male reproductive system and spermatogenesis in the red claw crayfish, *Cherax quadricarinatus*. J. Crust. Biol. 31(2), 223-230. https://doi.org/https://doi.org/10.1651/10-3342.1.

Anderson, W.A., Ellis, R.A., 1967. Cytodifferentiation of the crayfish spermatozoon: acrosome formation, transformation of mitochondria and development of microtubules. Z Zellforsch Mikrosk Anat 77(1), 80-94. https://doi.org/https://doi.org/10.1007/bf00336700.

Ashizawa, K., Wishart, G.J., Katayama, S., Takano, D., Maeda, M., Arakawa, E., Tsuzuki, Y., 2006. Effects of calpain and Rho-kinase inhibitors on the acrosome reaction and motility of fowl spermatozoa *in vitro*. Reproduction. 131(1), 71. https://doi.org/https://doi.org/10.1530/rep.1.00588.

Aungsuchawan, S., Browdy, C.L., Withyachumnarnkul, B., 2011. Sperm capacitation of the shrimp *Litopenaeus vannamei*. Aquac. Res 42(2), 188-195. https://doi.org/https://doi.org/10.1111/j.1365-2109.2010.02579.x.

Bart, A.N., Choosuk, S., Thakur, D.P., 2006. Spermatophore cryopreservation and artificial insemination of black tiger shrimp, *Penaeus monodon* (Fabricius). Aquac. Res. 37(5), 523-528. https://doi.org/https://doi.org/10.1111/j.1365-2109.2006.01460.x.

Bart, A.N., Dunham, R.A., 1996. Effects of sperm concentration and egg number on fertilization efficiency with channel catfish (*Ictalurus punctatus*) eggs and blue catfish (*I. furcatus*) spermatozoa. Theriogenology 45(3), 673-682. https://doi.org/https://doi.org/10.1016/0093-691X(95)00413-3.

Bäumer, C., Fisch, E., Wedler, H., Reinecke, F., Korfhage, C., 2018. Exploring DNA quality of single cells for genome analysis with simultaneous whole-genome amplification. Sci Rep 8(1), 7476-7476. https://doi.org/https://doi.org/10.1038/s41598-018-25895-7.

Beach, D., Talbot, P., 1987. Ultrastructural comparison of sperm from the crayfishes *Cherax tenuimanus* and *Cherax albidus*. J. Crust. Biol. 7(2), 205-218. https://doi.org/https://doi.org/10.2307/1548602.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J.K., Peixoto, S., Robeck, T.R., Cabrita, E., 2019. Sperm handling in aquatic animals for artificial reproduction. Theriogenology 133, : 161-178. https://doi.org/https://doi.org/10.1016/j.theriogenology.2019.05.004.

Best, B.P., 2015. Cryoprotectant toxicity: facts, issues, and questions. Rejuvenation Res 18(5), 422-436. https://doi.org/https://dx.doi.org/10.1089%2Frej.2014.1656.

Bhattacharya, S., 2018. Cryoprotectants and their usage in cryopreservation process, in: Bozkurt, Y. (Ed.), Cryopreservation Biotechnology in Biomedical and Biological Sciences. IntechOpen, IntechOpen Limited Headquarters, The Shard, 25th floor, 32 London Bridge Street, London, SE1 9SG, United Kingdom, pp. 7-19.

Bhavanishankar, S., Subramoniam, T., 1997. Cryopreservation of spermatozoa of the edible mud crab *Scylla serrata* (Forskal). J. Exp. Zool. 277(4), 326-336. https://doi.org/https://doi.org/10.1002/(SICI)1097-010X(19970301)277:4%3C326::AID-JEZ6%3E3.0.CO;2-R.

Binet, M.T., Doyle, C.J., Williamson, J.E., Schlegel, P., 2014. Use of JC-1 to assess mitochondrial membrane potential in sea urchin sperm. J. Exp. Mar. Bio 452, : 91-100. https://doi.org/https://doi.org/10.1016/j.jembe.2013.12.008.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165(3), 535-548. https://doi.org/https://doi.org/10.1016/j.ygcen.2009.02.011.

Bombardelli, R.A., Sanches, E.A., Baggio, D.M., Sykora, R.M., Souza, B.E.d., Tessaro, L., Piana, P.A., 2013. Effects of the spermatozoa: oocyte ratio, water volume and water temperature on artificial fertilization and sperm activation of cascudo-preto. R. Bras. Zootec. 42(1), 1-6. https://doi.org/https://doi.org/10.1590/S1516-35982013000100001.

Boulais, M., Soudant, P., Le Goïc, N., Quéré, C., Boudry, P., Suquet, M., 2015. Involvement of mitochondrial activity and OXPHOS in ATP synthesis during the motility phase of spermatozoa in the Pacific Oyster, *Crassostrea gigas*. Biol. Reprod. 93(5). https://doi.org/https://doi.org/10.1095/biolreprod.115.128538.

Braga, A., Lopes, D., Magalhães, V., Klosterhoff, M.C., Romano, L.A., Poersch, L.H., Wasielesky, W., 2018. Hemocytic melanization in shrimp spermatophores. Aquaculture 486, : 64-67. https://doi.org/https://doi.org/10.1016/j.aquaculture.2017.12.018.

Braga, A., Nakayama, C.L., Poersch, L., Wasielesky, W., 2013. Unistellate spermatozoa of decapods: comparative evaluation and evolution of the morphology. Zoomorphology 132(3), 261-284. https://doi.org/https://doi.org/10.1007/s00435-013-0187-2.

Braga, A., Suita de Castro, L.A., Poersch, L.H., Wasielesky, W., 2014. Spermatozoal capacitation of pink shrimp *Farfantepenaeus paulensis*. Aquaculture 430, : 207-210. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.04.011.

Braga, A.L., Nakayama, C.L., Martins, J.G., Colares, E.P., Wasielesky, W., 2010. Spermatophore quality of the pink shrimp *Farfantepenaeus paulensis* (Decapoda, Dendrobranchiata) broodstock fed with different maturation diets. Aquaculture 307(1), 44-48. https://doi.org/https://doi.org/10.1016/j.aquaculture.2010.07.010.

Braude, P., Bolton, V., Moore, S., 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 332(6163), 459-461. https://doi.org/https://doi.org/10.1038/332459a0.

Bray, W.A., Lawrence, A.L., 1998. Male viability determinations in *Penaeus vannamei*: evaluation of short-term storage of spermatophores up to 36 h and comparison of Ca-free saline and seawater as sperm homogenate media. Aquaculture 160(1), 63-67. https://doi.org/https://doi.org/10.1016/S0044-8486(97)00225-1.

Breitbart, H., 2002. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. Mol. Cell. Endocrinol. 187(1), 139-144. https://doi.org/https://doi.org/10.1016/S0303-7207(01)00704-3.

Bugnot, A.B., López Greco, L.S., 2009a. Sperm production in the red claw crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). Aquaculture 295(3), 292-299. https://doi.org/https://doi.org/10.1016/j.aquaculture.2009.07.021.

Bugnot, A.B., López Greco, L.S., 2009b. Structural alterations in the male reproductive system of the freshwater crayfish, *Cherax quadricarinatus* (Decapoda, Parastacidae). J. Invertebr. Pathol. 102(2), 160-166. https://doi.org/https://doi.org/10.1016/j.jip.2009.08.004.

Bungum, M., Bungum, L., Giwercman, A., 2011. Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. *Asian J Androl*. 13(1), 69-75. https://doi.org/https://doi.org/10.1038/aja.2010.73.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V., 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432, 389-401. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.04.034.

Cabrita, E., Robles, V., Herráez, M.P., 2009. Sperm quality assessment, in: Cabrita, E., Robles, V., Herráez, M.P. (Eds.), Methods in Reproductive Aquaculture: Marine andFreshwater Species. Biology Series. CRC Press (Taylor and Francis Group), Boca Raton, Florida, USA, pp. 93-148.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M.P., 2010. Cryopreservation of fish sperm: applications and perspectives. J. Appl. Ichthyol 26(5), 623-635. https://doi.org/https://doi.org/10.1111/j.1439-0426.2010.01556.x.

Castelo-Branco, T., Batista, A.M., Guerra, M.M.P., Soares, R., Peixoto, S., 2015. Sperm vitrification in the white shrimp *Litopenaeus vannamei*. Aquaculture 436, : 110-113. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.11.005.

Castelo-Branco, T., Guerra, M.M.P., Soares, R., Peixoto, S., 2018. Sperm vitrification of *Litopenaeus vannamei*: effect of cryoprotectant solutions on sperm viability and

spawning quality after artificial insemination. Aquac. Int. 26(3), 913-920. https://doi.org/https://doi.org/10.1007/s10499-018-0258-y.

Ceballos-Vázquez, B.P., Rosas, C., Racotta, I.S., 2003. Sperm quality in relation to age and weight of white shrimp *Litopenaeus vannamei*. Aquaculture 228(1), 141-151. https://doi.org/https://doi.org/10.1016/S0044-8486(03)00322-3.

Chao, N.-H., Chen, Y.-N., Shieh, W.Y., Lin, K.-J., 2009. Cryopreservation of spermatophores in the white shrimp (*Litopenaeus vannamei*). J. Taiwan Fish. Res. 17(2), 67-76.

Chapman, F.A., 2016. A semen extender for the short-term storage of fish sperm, Fisheries and Aquatic Sciences Department. Fisheries and Aquatic Sciences Department, UF/IFAS Extension, Gainesville, FL 32611., pp. 1-3.

Chaves, T.C.B., Fernandes, A.B., Mello, M.R.B.d., Oshiro, L.M.Y., 2014. Cryopreservation and sperm storage of the white shrimp. Bol. Inst. Pesca 40(1), 17-22.

Chávez, J.C., De la Vega-Beltrán, J.L., José, O., Torres, P., Nishigaki, T., Treviño, C.L., Darszon, A., 2018. Acrosomal alkalization triggers Ca2+ release and acrosome reaction in mammalian spermatozoa. J. Cell. Physiol. 233(6), 4735-4747. https://doi.org/https://doi.org/10.1002/jcp.26262.

Cho, C.-L., Agarwal, A., 2017. Role of sperm DNA fragmentation in male factor infertility: A systematic review. Arab J. Urol. 16(1), 21-34. https://doi.org/https://doi.org/10.1016/j.aju.2017.11.002.

Chow, S., 1982. Artificial Insemination Using Preserved Spermatophores in the Palaemonid Shrimp *Macrobrachium rosenbergii*. Nippon Suisan Gakkaishi. 48(12), 1693-1695. https://doi.org/http://dx.doi.org/10.2331/suisan.48.1693.

Chow, S., Tam, Y., Ogasawara, Y., 1985. Cryopreservation of spermatophore of the freshwater shrimp, *Macrobrachium resenbergii*. Biol. Bull. 168(3), 471-475. https://doi.org/https://doi.org/10.2307/1541526.

Clark Jr, W.H., Griffin, F.J., 1988. The morphology and physiology of the acrosome reaction in the sperm of the decapod, *Sicyonia ingentis*. Dev. Growth & Differ. 30(5), 451-462. https://doi.org/https://doi.org/10.1111/j.1440-169X.1988.00451.x.

Clark, W.H., Talbot, P., Neal, R.A., Mock, C.R., Salser, B.R., 1973. *In vitro* fertilization with non-motile spermatozoa of the brown shrimp *Penaeus aztecus*. Mar. Biol. 22(4), 353-354. https://doi.org/https://doi.org/10.1111/j.1440-169X.1988.00451.x.

Cloud, J., Patton, S., 2009. Basic principles of fish spermatozoa cryopreservation, in: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in Reproductive Aquaculture: Marine and Freshwater Species. CRC Press, Taylor & Francis Group, 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL, pp. 219-295.

Cossarizza, A., Salvioli, S., 2000. Flow Cytometric Analysis of Mitochondrial Membrane Potential Using JC-1. Curr. Protoc. Cytom. 13(1), 9.14.11-19.14.17. https://doi.org/https://doi.org/10.1002/0471142956.cy0914s13.

Costa, T.V.d., Oshiro, L.M.Y., Mattos, L.A.d., Lopez Greco, L.S., Melo, E.P., Flor, H.d.R., 2017. Toxicity, freezing and cold storage tests of native species semen. Bol. Inst. Pesca 43(3), 334-346. https://doi.org/https://doi.org/10.20950/1678-2305.2017v43n3p334.

Dar, S., Grover, S.A., Moskovtsev, S.I., Swanson, S., Baratz, A., Librach, C.L., 2013. *In vitro* fertilization–intracytoplasmic sperm injection outcome in patients with a markedly high DNA fragmentation index (>50%). Fertil. Steril. 100(1), 75-80. https://doi.org/https://doi.org/10.1016/j.fertnstert.2013.03.011.

Darżynkiewicz, Z., Traganos, F., Sharpless, T., Melamed, M.R., 1975. Thermal denaturation of DNA *in situ* as studied by acridine orange staining and automated cytofluorometry. Exp. Cell Res. 90(2), 411-428. https://doi.org/https://doi.org/10.1016/0014-4827(75)90331-6.

De Baulny, B.O., Le Vern, Y., Kerboeuf, D., Maisse, G., 1997. Flow cytometric evaluation of mitochondrial activity and membrane integrity in fresh and cryopreserved rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Cryobiology 34(2), 141-149. https://doi.org/https://doi.org/10.1006/cryo.1996.1992.

Devaux, A., Fiat, L., Gillet, C., Bony, S., 2011. Reproduction impairment following paternal genotoxin exposure in brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*). Aquat. Toxicol. 101(2), 405-411. https://doi.org/https://doi.org/10.1016/j.aquatox.2010.11.017.

Diggles, B.K., 2019. Review of some scientific issues related to crustacean welfare. ICES J. Mar. Sci 76(1), 66-81. https://doi.org/https://doi.org/10.1093/icesjms/fsy058.

Diwan, A.D., Joseph, S., 1998. Cryopreservation of spermatophores of the marine shrimp P*enaeus indicus H*. Milne Edwards, in: Fish Genetics and Biodiversity Conservation. Nature Conservators. Mussafarnagar, pp. 243-251.

Díz, A.C., Gimenez, A.V.F., Harán, N.S., Fenucci, J.L., 2001. Reproductive Performance of Male Argentine Red Shrimp *Pleoticus muelleri* Bate (Decapoda, Penaeoidea) in Culture Conditions. J. World Aquac. Soc. 32(2), 236-242. https://doi.org/https://doi.org/10.1111/j.1749-7345.2001.tb01101.x.

Dudenhausen, E.E., Talbot, P., 1983. An ultrastructural comparison of soft and hardened spermatophores from the crayfish *Pacifastacus leniusculus* Dana. Can. J. Zool. 61(1), 182-194. https://doi.org/https://doi.org/10.1139/z83-023.

Dupré, E., Goldstein, M., Palma, S., 2012. Acrosome reaction of *Cancer setosus* Molina, 1782 (Decapoda: Brachyura). J. Crust. Biol. 32(2), 181-189. https://doi.org/https://doi.org/10.1163/193724011X615505.

Dupre, E., Schatten, G., 1993. Microfilament and DNA localisation in lobster and crayfish spermatozoa. Biol. Res. 26, 529-537.

Dupré, E.M., Barros, C., 2011. *In vitro* fertilization of the rock shrimp, *Rhynchocinetes typus* (Decapoda, Caridea): a review. Biol. Res. 44(2), 125-133. https://doi.org/http://dx.doi.org/10.4067/S0716-97602011000200003

Elliott, G.D., Wang, S., Fuller, B.J., 2017. Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology 76, 74-91. https://doi.org/https://doi.org/10.1016/j.cryobiol.2017.04.004.

Erenpreisa, J., Erenpreiss, J., Freivalds, T., Slaidina, M., Krampe, R., Butikova, J., Ivanov, A., Pjanova, D., 2003. Toluidine Blue Test for Sperm DNA Integrity and Elaboration of Image Cytometry Algorithm. Cytometry A 52(1), 19-27. https://doi.org/https://doi.org/10.1002/cyto.a.10015.

Erkan, M., Tunalı, Y., Sancar-Bas, S., 2009. Male reproductive system morphology and spermatophore formation in *Astacus Leptodactylus* (Eschscholtz, 1823) (Decapoda: Astacidae). J. Crust. Biol. 29(1), 42-50. https://doi.org/https://doi.org/10.1651/08-2991.1.

Erraud, A., Bonnard, M., Duflot, A., Geffard, A., Danger, J.-M., Forget-Leray, J., Xuereb, B., 2018. Assessment of sperm quality in palaemonid prawns using Comet assay: methodological optimization. Environ. Sci. Pollut. Res. 25(12), 11226-11237. https://doi.org/https://doi.org/10.1007/s11356-017-8754-6.

Erraud, A., Bonnard, M., Geffard, O., Coulaud, R., Poret, A., Duflot, A., Forget-Leray, J., Geffard, A., Xuereb, B., 2019. Signification of DNA integrity in sperm of *Palaemon serratus* (Pennant 1777): Kinetic responses and reproduction impairment. Mar. Environ. Res. 144, 130-140. https://doi.org/https://doi.org/10.1016/j.marenvres.2019.01.005.

Evenson, D.P., 1999. Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. Reprod. Fertil. Dev. 11(1), 1-16. https://doi.org/https://doi.org/10.1071/RD98023.

Evenson, D.P., 2016. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Anim. Reprod. Sci. 169, : 56-75. https://doi.org/https://doi.org/10.1016/j.anireprosci.2016.01.017.

Evenson, D.P., Darzynkiewicz, Z., Melamed, M.R., 1982. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J. Histochem. Cytochem. 30(3), 279-280. https://doi.org/https://doi.org/10.1177/30.3.6174566.

Fang, D.-A., Wang, Q., He, L., Wang, J., Wang, Y., 2012. Characterization of heat shock protein 70 in the red claw crayfish (*Cherax quadricarinatus*): Evidence for its role in regulating spermatogenesis. Gene 492(1), 138-147. https://doi.org/https://doi.org/10.1016/j.gene.2011.10.036.

Fang, D.-A., Wang, Y., Wang, J., Liu, L.-H., Wang, Q., 2013. Characterization of *Cherax quadricarinatus* prohibitin and its potential role in spermatogenesis. Gene 519(2), 318-325. https://doi.org/https://doi.org/10.1016/j.gene.2013.02.003.

FAO-FIGIS, 2019. Global fisheries and aquaculture production 1950 - 2017, Fisheries Global Information System (FIGIS). FI Institutional Websites. Statistics and Information Branch. Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153, Rome.

FAO, 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action, The State of World Fisheries and Aquaculture (SOFIA). FAO, Rome, Italy, p. 244.

Farhadi, A., Harlıoğlu, A.G., 2019. Molecular and cellular biology of the crayfish spermatozoon: Toward development of artificial reproduction in aquaculture. Rev. Fish. Sci. Aquac. 27(2), 198-214. https://doi.org/https://doi.org/10.1080/23308249.2018.1552246.

Farhadi, A., Harlıoglu, M.M., 2018. Elevated water temperature impairs gamete production in male narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Knowl. Manag. Aquat. Ecosyst. 419(40), 1-6. https://doi.org/http://dx.doi.org/10.1051/kmae/2018029.

Farhadi, A., Harlıoğlu, M.M., 2019. The annual cycle of spermatozoa content in the vas deferens and some reproductive parameters in the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Aquac. Rep. 13(100185), 1-5. https://doi.org/https://doi.org/10.1016/j.aqrep.2019.100185.

Farhadi, A., Harlıoğlu, M.M., Gür, S., 2019a. Artificial extrusion of spermatophores for insemination of the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Aquac. Rep. 14(100200), 1-5. https://doi.org/https://doi.org/10.1016/j.aqrep.2019.100200.

Farhadi, A., Harlıoğlu, M.M., Gür, S., Acısu, T.C., 2018. Optimization of the incubation time and temperature for spermatozoa extraction in freshwater crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Anim. Reprod. Sci. 193, : 153-157. https://doi.org/https://doi.org/10.1016/j.anireprosci.2018.04.063.

Farhadi, A., Harlıoğlu, M.M., Harlıoğlu, A.G., 2019b. The relationship between weight and length of male broodstock with gamete production in the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Aquac. Res. 50(4), 1380-1383. https://doi.org/https://doi.org/10.1111/are.13982.

Fatihah, S.N., Jasmani, S., Abol-Munafi, A.B., Noorbaiduri, S., Muhd-Farouk, H., Ikhwanuddin, M., 2016. Development of a sperm cryopreservation protocol for the mud spiny lobster, *Panulirus polyphagus*. Aquaculture 462, 56-63. https://doi.org/https://doi.org/10.1016/j.aquaculture.2016.04.025.

Fatihah, S.N., Simon, S.C., Hashim, H.H., Harman, M.-F., Chen, C.A., 2018. Sperm viability and quantity of mud crab, *Scylla tranquebarica* in different cryoprotectants. Aquac Aquar Conserv Legis 11(4), 1098-1106.

Favret, K.P., Lynn, J.W., 2010. Flow-cytometric analyses of viability biomarkers in pesticide-exposed sperm of three aquatic invertebrates. Arch. Environ. Contam. Toxicol. 58(4), 973-984. https://doi.org/https://doi.org/10.1007/s00244-009-9410-z.

Feng, T., 2018. Spermatogenesis and Sperm Assessment in the Black Tiger Prawn, *Penaeus monodon*, School of Agriculture and Food Sciences. University of Queensland, Queensland, Australia, p. 195.

Feng, T., Gosálvez, J., Lopez-Fernandez, C., Arroyo, F., Paterson, B., Johnston, S., 2018a. Sperm chromatin dispersion test (SCDt) for the assessment of sperm DNA fragmentation in black tiger prawn, *Penaeus monodon*. Aquaculture 491, : 281-288. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.03.041.

Feng, T., Paterson, B., Coman, G., Johnston, S., 2019. Evaluating the impact of moulting and chilled storage of spermatophores on the integrity of plasma membrane, acrosome and DNA of black tiger prawn (*Penaeus monodon*) spermatozoa. Aquac. Res. 50(1), 226-235. https://doi.org/https://doi.org/10.1111/are.13888.

Feng, T., Paterson, B., Johnston, S., 2018b. A morphological study of the male reproductive tract, post-testicular acrosome maturation and spermatophore formation in the black tiger prawn (*Penaeus monodon*). J. Morphol. 279(9), 1290-1300. https://doi.org/https://doi.org/10.1002/jmor.20869.

Feng, T., Paterson, B., Johnston, S.D., 2017. New insights into the spermatogenesis of the black tiger prawn, *Penaeus monodon*. J. Morphol. 278(5), 689-703. https://doi.org/https://doi.org/10.1002/jmor.20664.

Fernández-Díez, C., González-Rojo, S., Lombó, M., Herráez, M.P., 2016. Impact of sperm DNA damage and oocyte-repairing capacity on trout development. Reproduction. 152(1), 57-67. https://doi.org/https://doi.org/10.1530/REP-16-0077.

Fernández-Díez, C., Herráez, M.P., 2018. Changes in transcriptomic profile of trout larvae obtained with frozen sperm. Aquaculture 492, : 306-320. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.04.022.

Fernández, J.L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., LaFromboise, M., De Jonge, C., 2005. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil. Steril. 84(4), 833-842. https://doi.org/https://doi.org/10.1016/j.fertnstert.2004.11.089.

Fernández, J.L., Muriel, L., Rivero, M.T., Goyanes, V., Vazquez, R., Alvarez, J.G., 2003. The Sperm Chromatin Dispersion Test: A simple method for the determination of Sperm DNA Fragmentation. J. Androl. 24(1), 59-66. https://doi.org/https://doi.org/10.1002/j.1939-4640.2003.tb02641.x.

Figueroa, E., Farias, J.G., Lee-Estevez, M., Valdebenito, I., Risopatrón, J., Magnotti, C., Romero, J., Watanabe, I., Oliveira, R.P.S., 2018. Sperm cryopreservation with supplementation of α-tocopherol and ascorbic acid in freezing media increase sperm function and fertility rate in Atlantic salmon (*Salmo salar*). Aquaculture 493, : 1-8. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.04.046.

Figueroa, E., Lee-Estévez, M., Valdebenito, I., Farías, J.G., Romero, J., 2020. Potential biomarkers of DNA quality in cryopreserved fish sperm: impact on gene expression and embryonic development. Rev. Aquac. 12(1), 382-391. https://doi.org/https://doi.org/10.1111/raq.12323.

Figueroa, E., Lee-Estevez, M., Valdebenito, I., Watanabe, I., Oliveira, R.P.S., Romero, J., Castillo, R.L., Farías, J.G., 2019. Effects of cryopreservation on mitochondrial function and sperm quality in fish. Aquaculture 511(634190), 1-13. https://doi.org/https://doi.org/10.1016/j.aquaculture.2019.06.004.

Figueroa, E., Valdebenito, I., Farias, J.G., 2016. Technologies used in the study of sperm function in cryopreserved fish spermatozoa. Aquac. Res. 47(6), 1691-1705. https://doi.org/https://doi.org/10.1111/are.12630.

Figueroa, E., Valdebenito, I., Zepeda, A.B., Figueroa, C.A., Dumorné, K., Castillo, R.L., Farias, J.G., 2017. Effects of cryopreservation on mitochondria of fish spermatozoa. Rev. Aquac. 9(1), 76-87. https://doi.org/https://doi.org/10.1111/raq.12105.

Fraser, L., Strzezek, J., 2004. The use of comet assay to assess DNA integrity of boar spermatozoa following liquid preservation at 5 degrees C and 16 degrees C. Folia Histochem. Cytobiol. 42(1), 49-55.

Fraser, L.R., 1995. Cellular biology of capacitation and the acrosome reaction. Hum. Reprod 10(suppl_1), 22-30. https://doi.org/https://doi.org/10.1093/humrep/10.suppl_1.22.

Fraser, L.R., 1998. Sperm capacitation and the acrosome reaction. Hum. Reprod. 13(suppl_1), 9-19. https://doi.org/https://doi.org/10.1093/oxfordjournals.humrep.a137524.

Friend, D.S., Rudolf, I., 1974. Acrosomal disruption in sperm. Freeze-fracture of altered membranes. J. Cell Biol. 63(2 Pt 1), 466-479. https://doi.org/https://doi.org/10.1083/jcb.63.2.466.

Galeotti, P., Bernini, G., Locatello, L., Sacchi, R., Fasola, M., Rubolini, D., 2012. Sperm traits negatively covary with size and asymmetry of a secondary sexual trait in a freshwater crayfish. *PLoS One* 7(8), e43771-e43771. https://doi.org/https://doi.org/10.1371/journal.pone.0043771.

Gao, D., Critser, J.K., 2000. Mechanisms of cryoinjury in living cells. ILAR J. 41(4), 187-196. https://doi.org/https://doi.org/10.1093/ilar.41.4.187.

Garner, D.L., Johnson, L.A., 1995. Viability assessment of mammalian sperm using SYBR-14 and Propidium iodide. Biol. Reprod. 53(2), 276-284. https://doi.org/https://doi.org/10.1095/biolreprod53.2.276.

Garner, D.L., Pinkel, D., Johnson, L.A., Pace, M.M., 1986. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol. Reprod. 34(1), 127-138. https://doi.org/https://doi.org/10.1095/biolreprod34.1.127.

Garner, D.L., Thomas, C.A., Joerg, H.W., DeJarnette, J.M., Marshall, C.E., 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 57(6), 1401-1406. https://doi.org/https://doi.org/10.1095/biolreprod57.6.1401.

Gillan, L., Evans, G., Maxwell, W.M.C., 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63(2), 445-457. https://doi.org/https://doi.org/10.1016/j.theriogenology.2004.09.024.

Gledhill, B.L., 1983. Cytometry of Deoxyribonucleic Acid Content and Morphology of Mammalian Sperm1, 2. Int. J. Dairy Sci. 66(12), 2623-2634. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(83)82136-5.

Goudeau, H., Goudeau, M., 1989. Electrical responses to fertilization and spontaneous activation in decapod crustacean eggs: Characteristics and role, in: Nuccitelli, R., Cherr, G.N., Clark, W.H. (Eds.), Mechanisms of Egg Activation. Springer US, Boston, MA, pp. 61-88.

Goudeau, M., Jacqueline, B., 1982. Fertilization in a crab. II. Cytological aspects of the cortical reaction and fertilization envelope elaboration. Tissue Cell 14(2), 273-282. https://doi.org/https://doi.org/10.1016/0040-8166(82)90025-8.

Gould, M.C., Stephano, J.L., 2003. Polyspermy prevention in marine invertebrates. Microsc. Res. Tech. 61(4), 379-388. https://doi.org/https://doi.org/10.1002/jemt.10351.

Graham, J.K., Kunze, E., Hammerstedt, R.H., 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol. Reprod. 43(1), 55-64. https://doi.org/https://doi.org/10.1095/biolreprod43.1.55.

Gravance, C.G., Garner, D.L., Baumber, J., Ball, B.A., 2000. Assessment of equine sperm mitochondrial function using JC-1. Theriogenology 53(9), 1691-1703. https://doi.org/https://doi.org/10.1016/S0093-691X(00)00308-3.

Gregati, R.A., Fransozo, V., López-Greco, L.S., Negreiros-Fransozo, M.L., Bauer, R., 2014. Functional morphology of the reproductive system and sperm transfer in *Stenopus hispidus* (Crustacea: Decapoda: Stenopodidea), and their relation to the mating system. Invertebr. Biol. 133(4), 381-393. https://doi.org/https://doi.org/10.1111/ivb.12067.

Griffin, F.J., Clark Jr., W.H., 1990. Induction of acrosomal filament formation in the sperm of *Sicyonia ingentis*. J. Exp. Zool. 254(3), 296-304. https://doi.org/https://doi.org/10.1002/jez.1402540308.

Guthrie, H.D., Woods, L.C., Long, J.A., Welch, G.R., 2008. Effects of osmolality on inner mitochondrial transmembrane potential and ATP content in spermatozoa recovered from the testes of striped bass (*Morone saxatilis*). Theriogenology 69(8), 1007-1012. https://doi.org/https://doi.org/10.1016/j.theriogenology.2008.01.021.

Gwo, J.-C., 2000. Cryopreservation of aquatic invertibrate semen: a review. Aquac Res 31(3), 259-271. https://doi.org/https://doi.org/10.1046/j.1365-2109.2000.00462.x.

Gwo, J.-C., 2009. Methods for sperm collection, in: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in Reproductive Aquaculture. Taylor & Francis Group, United States of America, pp. 81-86.

Haldar, C., Kumar, S., Ram, R., 2018. Artificial insemination and its importance in marine crustaceans: A review. Mar. Biol. & Oceanogr. 1(4), 1-3. https://doi.org/http://dx.doi.org/10.31031/eimbo.2018.01.000524.

Harlıoğlu, M.M., Farhadi, A., 2017. Factors affecting the reproductive efficiency in crayfish: implications for aquaculture. Aquac. Res. 48(5), 1983-1997. https://doi.org/https://doi.org/10.1111/are.13263.

Harlıoğlu, M.M., Farhadi, A., Gür, S., 2018. Determination of sperm quality in decapod crustaceans. Aquaculture 490, : 185-193. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.02.031.

Harlioğlu, M.M., Köprücü, K., Harlioğlu, A.G., Yonar, S.M., Duran, T.Ç., Çakmak, M.N., Aksu, Ö., Özcan, S., Kutluyer, F., Gündoğdu, H., 2013. Effect of dietary n-3 series

fatty acids on sperm production in the freshwater crayfish, *Astacus leptodactylus* (Eschscholtz) (Astacidae). Aquac. Int. 21(2), 273-282. https://doi.org/https://doi.org/10.1007/s10499-012-9549-x.

Harlioğlu, M.M., Kutluyer, F., Gür, S., 2012. An investigation on the sperm number and reproductive parameters of males in wild caught freshwater crayfish (*Astacus leptodactylus*, Eschscholtz). Anim. Biol. 62(4), 409-418. https://doi.org/https://doi.org/10.1163/157075612X638559.

Harris, S.E.G., Sandifer, P.A., 1986. Sperm production and the effects of electrically induced spermatophore expulsion in the prawn *Macrobrachium Rosenbergii* (De Man). J. Crust. Biol. 6(4), 633-647. https://doi.org/https://doi.org/10.1163/193724086X00433.

Harzsch, S., Miller, J., Benton, J., Beltz, B., 1999. From embryo to adult: Persistent neurogenesis and apoptotic cell death shape the lobster deutocerebrum. J. Neurosci. 19(9), 3472. https://doi.org/https://doi.org/10.1523/JNEUROSCI.19-09-03472.1999.

Hinsch, G.W., 1993. Ultrastructure of spermatogonia, spermatocytes, and sertoli cells in the testis of the crayfish, *Procambarus paeninsulanus*. Tissue Cell 25(5), 737-742. https://doi.org/https://doi.org/10.1016/0040-8166(93)90054-O.

Hirohashi, N., Yanagimachi, R., 2018. Sperm acrosome reaction: its site and role in fertilization. Biol. Reprod. 99(1), 127-133. https://doi.org/https://doi.org/10.1093/biolre/ioy045.

Hobbs, C.A., Recio, L., Winters, J., Witt, K.L., 2020. Use of frozen tissue in the comet assay for the evaluation of DNA damage. J. Vis. Exp. 157, : 1-9. https://doi.org/https://doi.org/10.3791/59955.

Hossain, M.S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A.P., Rodriguez-Martinez, H., 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian J. Androl. 13(3), 406-419. https://doi.org/https://doi.org/10.1038/aja.2011.15.

Hou, X.-L., Mao, Q., He, L., Gong, Y.-N., Qu, D., Wang, Q., 2010. Accessory sex gland proteins affect spermatophore digestion rate and spermatozoa acrosin activity in *Eriocheir Sinensis*. J. Crust. Biol. 30(3), 435-440. https://doi.org/https://doi.org/10.1651/09-3177.1.

Ishida, T., Talbot, P., Kooda-Cisco, M., 1986. Technique for the long-term storage of lobster (*Homarus*) spermatophores. Gamete Res. 14(3), 183-195. https://doi.org/https://doi.org/10.1002/mrd.1120140302.

Jackson, P., Pedersen, L.M., Kyjovska, Z.O., Jacobsen, N.R., Saber, A.T., Hougaard, K.S., Vogel, U., Wallin, H., 2013. Validation of freezing tissues and cells for analysis of DNA strand break levels by comet assay. Mutagenesis 28(6), 699-707. https://doi.org/https://dx.doi.org/10.1093%2Fmutage%2Fget049.

Janny, L., Menezo, Y.J.R., 1994. Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. Mol. Reprod. Dev. 38(1), 36-42. https://doi.org/https://doi.org/10.1002/mrd.1080380107.

Jerry, D.R., 2001. Electrical stimulation of spermatophore extrusion in the freshwater yabby (*Cherax destructor*). Aquaculture 200(3), 317-322. https://doi.org/https://doi.org/10.1016/S0044-8486(01)00511-7.

Jeyalectumie, C., Subramoniam, T., 1989. Cryopreservation of spermatophores and seminal plasma of the edible crab *Scylla serrata*. Biol. Bull. 177(2), 247-253. https://doi.org/https://doi.org/10.2307/1541940.

Jones, C.M., 1995. Production of juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae) I. Development of hatchery and nursery procedures. Aquaculture 138(1), 221-238. https://doi.org/https://doi.org/10.1016/0044-8486(95)00068-2.

Kang, X., Li, G., Mu, S., Guo, M., Ge, S., 2009. Acrosome reaction of Chinese mitten-handed crab *Eriocheir sinensis* (Crustacea: Decapoda) spermatozoa: Promoted by long-term cryopreservation. Aquaculture 295(3), 195-199. https://doi.org/https://doi.org/10.1016/j.aquaculture.2009.06.041.

Kanokpan, W., Kornnika, K., Supatra Somapa, G., Prasert, M., Boonsirm, W., 2003. Time-course and levels of apoptosis in various tissues of black tiger shrimp *Penaeus monodon* infected with white-spot syndrome virus. Dis. Aquat. Org. 55(1), 3-10. https://doi.org/https://doi.org/10.3354/dao055003.

Khalaila, I., Manor, R., Weil, S., Granot, Y., Keller, R., Sagi, A., 2002. The eyestalk–androgenic gland–testis endocrine axis in the crayfish *Cherax quadricarinatus*. Gen. Comp. Endocrinol. 127(2), 147-156. https://doi.org/https://doi.org/10.1016/S0016-6480(02)00031-X.

Khalaila, I., Weil, S., Sagi, A., 1999. Endocrine balance between male and female components of the reproductive system in intersex *Cherax quadricarinatus* (Decapoda: Parastacidae). J. Exp. Zool. 283(3), 286-294. https://doi.org/https://doi.org/10.1002/(SICI)1097-010X(19990215)283:3%3C286::AID-JEZ6%3E3.0.CO;2-R.

Kooda-Cisco, M.J., Talbot, P., 1983. A technique for electrically stimulating extrusion of spermatophores from the lobster, *Homarus americanus*. Aquaculture 30(1), 221-227. https://doi.org/https://doi.org/10.1016/0044-8486(83)90164-3.

Kordan, W., Fraser, L., Wysocki, P., Strzeżek, R., Lecewicz, M., Mogielnicka-Brzozowska, M., Dziekońska, A., Soliwoda, D., Koziorowska-Gilun, M., 2013. Semen quality assessments and their significance in reproductive technology. Pol. J. Vet. Sci. 16(4), 823-833. https://doi.org/https://doi.org/10.2478/pjvs-2013-0117.

Kornnika, K., Chumporn, S., Flegel, T.W., Sukathida, U., Boonsirm, W., 2002. Evidence for apoptosis correlated with mortality in the giant black tiger shrimp *Penaeus monodon* infected with yellow head virus. Dis. Aquat. Org. 48(2), 79-90. https://doi.org/https://doi.org/10.3354/dao048079.

Kouba, A., Niksirat, H., Bláha, M., 2015. Comparative ultrastructure of spermatozoa of the redclaw *Cherax quadricarinatus* and the yabby *Cherax destructor* (Decapoda, Parastacidae). Micron 69, : 56-61. https://doi.org/https://doi.org/10.1016/j.micron.2014.11.002.

Kowaltowski, A.J., de Souza-Pinto, N.C., Castilho, R.F., Vercesi, A.E., 2009. Mitochondria and reactive oxygen species. Free Radic. Biol. Med. 47(4), 333-343. https://doi.org/https://doi.org/10.1016/j.freeradbiomed.2009.05.004.

Kruevaisayawan, H., Vanichviriyakit, R., Weerachatyanukul, W., Iamsaard, S., Withyachumnarnkul, B., Basak, A., Tanphaichitr, N., Sobhon, P., 2008. Induction of the acrosome reaction in Black Tiger Shrimp (*Penaeus monodon*) requires sperm trypsin-like enzyme activity. Biol. Reprod. 79(1), 134-141. https://doi.org/https://doi.org/10.1095/biolreprod.107.066316.

Kurtz, K., Ausió, J., Chiva, M., 2009. Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla.* Histones and nucleosome-like structures in decapod crustacean sperm nuclei previously described without SNBPs. Tissue Cell 41(5), 334-344. https://doi.org/https://doi.org/10.1016/j.tice.2009.02.003.

Lakshmi Pillai, S., Nasser, M., Sanil, N.K., 2014. Histology and ultrastructure of male reproductive system of the Indian spiny lobster *Panulirus homarus* (Decapoda: Palinuridae). Rev. Biol. Trop. 62(2), 533-541.

Leelatanawit, R., Uawisetwathana, U., Khudet, J., Klanchui, A., Phomklad, S., Wongtripop, S., Angthoung, P., Jiravanichpaisal, P., Karoonuthaisiri, N., 2014. Effects of polychaetes (*Perinereis nuntia*) on sperm performance of the domesticated black tiger shrimp (*Penaeus monodon*). Aquaculture 433, : 266-275. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.06.034.

Leung-Trujillo, J.R., Lawrence, A.L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65(3), 363-370. https://doi.org/https://doi.org/10.1016/0044-8486(87)90249-3.

Lewis, C., Ford, A.T., 2012. Infertility in male aquatic invertebrates: A review. Aquat. Toxicol. 120-121, : 79-89. https://doi.org/https://doi.org/10.1016/j.aquatox.2012.05.002.

Lezcano, M., Granja, C., Salazar, M., 2004. The use of flow cytometry in the evaluation of cell viability of cryopreserved sperm of the marine shrimp (*Litopenaeus vannamei*). Cryobiology 48(3), 349-356. https://doi.org/https://doi.org/10.1016/j.cryobiol.2004.03.003.

Li, G., Kang, X., Mu, S., 2010. Induction of acrosome reaction of spermatozoa in *Eriocheir sinensis* by low temperature. Cytotechnology 62(2), 101-107. https://doi.org/https://dx.doi.org/10.1007%2Fs10616-010-9264-2.

Li, N., Hou, Y.-H., Jing, W.-X., Dahms, H.-U., Wang, L., 2016. Quality decline and oxidative damage in sperm of freshwater crab *Sinopotamon henanense* exposed to lead. Ecotoxicol. Environ. Saf. 130, : 193-198. https://doi.org/https://doi.org/10.1016/j.ecoenv.2016.03.042.

Li, Y.-x., Zhou, L., Lv, M.-q., Ge, P., Liu, Y.-c., Zhou, D.-x., 2019. Vitrification and conventional freezing methods in sperm cryopreservation: A systematic review and meta-analysis. Eur. J. Obstet. Gynecol. Reprod. Biol. 233, : 84-92. https://doi.org/https://doi.org/10.1016/j.ejogrb.2018.11.028.

Liley, N.R., Tamkee, P., Tsai, R., Hoysak, D.J., 2002. Fertilization dynamics in rainbow trout (*Oncorhynchus mykiss*): effect of male age, social experience, and sperm concentration and motility on in vitro fertilization. Can. J. Fish. Aquat. Sci. 59(1), 144-152. https://doi.org/http://dx.doi.org/10.1139/f01-202.

Lindsay, L.L., Jr., W.H.C., 1992. Protease-induced formation of the sperm acrosomal filament. Develop. Growth & Differ. 34(2), 189-197. https://doi.org/https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1440-169X.1992.tb00008.x.

Liu, Q.H., Li, J., Zhang, S.C., Xiao, Z.Z., Ding, F.H., Yu, D.D., Xu, X.Z., 2007. Flow cytometry and ultrastructure of cryopreserved red seabream (*Pagrus major*) sperm. Theriogenology 67(6), 1168-1174. https://doi.org/https://doi.org/10.1016/j.theriogenology.2006.12.013.

López-Camps, J., Bargalló, R., Bozzo, M.G., Durfort, M., Fontarnau, R., 1981. The spermatogenesis of crustaceans. VII. Review of spermatozoon of the crayfish *Astacus astacus* (Malacostraca, Decapoda, Macrura, Reptantia). Gamete Res. 4(1), 65-82. https://doi.org/http://dx.doi.org/10.1002/mrd.1120040110.

López Greco, L.S., Lo Nostro, F.L., 2008. Structural changes in the spermatophore of the freshwater 'red claw' crayfish *Cherax quadricarinatus* (Von Martens, 1898) (Decapoda, Parastacidae). Acta Zool. (Stockholm) 89(2), 149-155. https://doi.org/http://dx.doi.org/10.1111/j.1463-6395.2007.00303.x.

López Greco, L.S., Vazquez, F., Rodríguez, E.M., 2007. Morphology of the male reproductive system and spermatophore formation in the freshwater 'red claw' crayfish *Cherax quadricarinatus* (Von Martens, 1898) (Decapoda, Parastacidae). Acta Zool. (Stockholm) 88(3), 223-229. https://doi.org/http://dx.doi.org/10.1111/j.1463-6395.2007.00269.x.

Ma, D.-D., Bi, L., Yang, W.-X., 2017. KIFC1 is essential for acrosome formation and nuclear shaping during spermiogenesis in the lobster *Procambarus clarkii*. Oncotarget 8(22), 36082-36098. https://doi.org/https://dx.doi.org/10.18632%2Foncotarget.16429.

Ma, D., Hou, Y., Du, L., Li, N., Xuan, R., Wang, F., Jing, W., Wang, L., 2013. Oxidative damages and ultrastructural changes in the sperm of freshwater crab *Sinopotamon henanense* exposed to cadmium. Ecotoxicol. Environ. Saf. 98, : 244-249. https://doi.org/https://doi.org/10.1016/j.ecoenv.2013.08.004.

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J.G., Valdebenito, I., Figueroa, E., 2018. Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. Rev. Aquac. 10(1), 15-25. https://doi.org/https://doi.org/10.1111/raq.12145.

Manicardi, G.C., Bianchi, P.G., Pantano, S., Azzoni, P., Bizzaro, D., Bianchi, U., Sakkas, D., 1995. Presence of Endogenous Nicks in DNA of Ejaculated Human Spermatozoa and its Relationship to Chromomycin A3 Accessibility. Biol. Reprod. 52(4), 864-867. https://doi.org/https://doi.org/10.1095/biolreprod52.4.864.

Manicardi, G.C., Bizzaro, D., Sakkas, D., 2011. Basic and Clinical Aspects of Sperm Chromomycin A3 Assay, in: Zini, A., Agarwal, A. (Eds.), Sperm Chromatin: Biological and Clinical Applications in Male Infertility and Assisted Reproduction. Springer New York, New York, NY, pp. 171-179.

Mansour, N., Lahnsteiner, F., Berger, B., 2004. Characterization of the testicular semen of the African catfish, *Clarias gariepinus* (Burchell, 1822), and its short-term storage. Aquac. Res. 35(3), 232-244. https://doi.org/https://doi.org/10.1111/j.1365-2109.2004.00993.x.

Marc, A.F., Guppy, J.L., Bauer, P., Mulvey, P., Jerry, D.R., Paris, D.B.B.P., 2021. Validation of advanced tools to evaluate sperm function in barramundi (*Lates calcarifer*). Aquaculture 531(735802), 1-14. https://doi.org/https://doi.org/10.1016/j.aquaculture.2020.735802.

Martinez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P., Rodriguez-Martinez, H., 2004. Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. Anim. Reprod. Sci 84(1), 121-133. https://doi.org/https://doi.org/10.1016/j.anireprosci.2003.12.006.

Mazur, P., Leibo, S.P., Chu, E.H.Y., 1972. A two-factor hypothesis of freezing injury: Evidence from Chinese hamster tissue-culture cells. Exp. Cell Res. 71(2), 345-355. https://doi.org/https://doi.org/10.1016/0014-4827(72)90303-5.

Memon, A.J., Talpur, A.D., Khan, M.I., Fariddudin, M.O., Safiah, J., Abol-Munafi, A.B., Ikhwanuddin, M., 2012. Optimization of Spermatophores Cryopreservation Protocol

of Banana Shrimp (*Penaeus merguiensis*) (De Man, 1888). J. Anim. Vet. Adv. 11(10), 1688-1704. https://doi.org/http://dx.doi.org/10.3923/javaa.2012.1688.1704.

Meunpol, O., Meejing, P., Piyatiratitivorakul, S., 2005. Maturation diet based on fatty acid content for male *Penaeus monodon* (Fabricius) broodstock. Aquac. Res. 36(12), 1216-1225. https://doi.org/https://doi.org/10.1111/j.1365-2109.2005.01342.x.

Morales-Ueno, K., Montaldo, H.H., Ortega, A.M., Paniagua-Chávez, C.G., Castillo-Juárez, H., 2013. An extender solution for the short-term storage of *Litopenaeus vannamei* sperm to be used in artificial insemination. Aquac. Res. 44(8), 1254-1258. https://doi.org/https://doi.org/10.1111/j.1365-2109.2012.03126.x.

Morales-Ueno, K., Paniagua-Chávez, C.G., Martínez-Ortega, A., Castillo-Juárez, H., Alfaro-Montoya, J., 2016. A simple method for short-term storage and transportation of spermatophores of Pacific white shrimp (*Litopenaeus vannamei*). Hidrobiologica 2016(1), 9-14. https://doi.org/https://doi.org/10.24275/uam/izt/dcbs/hidro/2016v26n1/Paniagua.

Mylonas, C.C., Duncan, N.J., Asturiano, J.F., 2017. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture 472, : 21-44. https://doi.org/https://doi.org/10.1016/j.aquaculture.2016.04.021.

Nabi, A., Khalili, M.A., Halvaei, I., Roodbari, F., 2014. Prolonged incubation of processed human spermatozoa will increase DNA fragmentation. Andrologia 46(4), 374-379. https://doi.org/https://doi.org/10.1111/and.12088.

Nakayama, C., Peixoto, S., Lopes, D., Vita, G., Krummenauer, D., Foes, G., Cavalli, R., Wasielesky, W., 2008. Manual and electric extrusion methods of spermatophores of wild breeders of *Farfantepenaeus paulensis* (Decapoda: Penaeidae). Cienc. Rural. 38(7), 2018-2022. https://doi.org/https://doi.org/10.1590/S0103-84782008000700034.

Nanshan, D., Luzheng, X., 1987. Induction of acrosome reaction of spermatozoa in the decapoda *Eriocheir sinensis*. Chin. J. Oceanol. Limnol. 5(2), 118-123. https://doi.org/https://ui.adsabs.harvard.edu/link_gateway/1987ChJOL...5..118D/doi:10.1007/BF02844996.

Nascimento, F.A.d., Zara, F.J., 2013. Development of the male reproductive system in *Callinectes ornatus* Ordway, 1863 (Brachyura: Portunidae). Nauplius 21, 161-177. https://doi.org/http://dx.doi.org/10.1590/S0104-64972013000200004.

Nesci, S., Spinaci, M., Galeati, G., Nerozzi, C., Pagliarani, A., Algieri, C., Tamanini, C., Bucci, D., 2020. Sperm function and mitochondrial activity: An insight on boar sperm metabolism. Theriogenology 144, : 82-88. https://doi.org/https://doi.org/10.1016/j.theriogenology.2020.01.004.

Niksirat, H., Andersson, L., James, P., Kouba, A., Kozák, P., 2014a. Proteomic profiling of the signal crayfish *Pacifastacus leniusculus* egg and spermatophore. Anim. Reprod. Sci. 149(3), 335-344. https://doi.org/https://doi.org/10.1016/j.anireprosci.2014.07.024.

Niksirat, H., James, P., Andersson, L., Kouba, A., Kozák, P., 2015a. Label-free protein quantification in freshly ejaculated versus post-mating spermatophores of the noble crayfish *Astacus astacus*. J. Proteom. 123, : 70-77. https://doi.org/https://doi.org/10.1016/j.jprot.2015.04.004.

Niksirat, H., Kouba, A., 2016. Subcellular localization of calcium deposits in the noble crayfish Astacus astacus spermatophore: Implications for post-mating spermatophore hardening and spermatozoon maturation. J. Morphol. 277(4), 445-452. https://doi.org/https://doi.org/10.1002/jmor.20509.

Niksirat, H., Kouba, A., Kozák, P., 2014b. Post-mating morphological changes in the spermatozoon and spermatophore wall of the crayfish *Astacus leptodactylus*: Insight into a non-motile spermatozoon. Anim. Reprod. Sci. 149(3), 325-334. https://doi.org/https://doi.org/10.1016/j.anireprosci.2014.07.017.

Niksirat, H., Kouba, A., Kozák, P., 2015b. Ultrastructure of egg activation and cortical reaction in the noble crayfish *Astacus astacus*. Micron 68, : 115-121. https://doi.org/https://doi.org/10.1016/j.micron.2014.09.010.

Niksirat, H., Kouba, A., Pšenička, M., Kuklina, I., Kozák, P., 2013. Ultrastructure of spermatozoa from three genera of crayfish *Orconectes*, *Procambarus* and *Astacus* (Decapoda: Astacoidea): New findings and comparisons. Zool. Anz. 252(2), 226-233. https://doi.org/https://doi.org/10.1016/j.jcz.2012.06.002.

Niksirat, H., Vancová, M., Andersson, L., James, P., Kouba, A., Kozák, P., 2016. Protein modification in the post-mating spermatophore of the signal crayfish *Pacifastacus leniusculus*: insight into the tyrosine phosphorylation in a non-motile spermatozoon. Anim. Reprod. Sci. 172, : 123-130. https://doi.org/https://doi.org/10.1016/j.anireprosci.2016.07.009.

Nimrat, S., Bart, A.N., Keatsaksit, A., Vuthiphandchai, V., 2008. Microbial flora of spermatophores from black tiger shrimp(*Penaeus monodon*) declines over long-term cryostorage. Aquaculture 274(2), 247-253. https://doi.org/https://doi.org/10.1016/j.aquaculture.2007.11.018.

Nimrat, S., Sangnawakij, T., Vuthiphandchai, V., 2005. Preservation of black tiger shrimp *Penaeus monodon* spermatophores by chilled storage. J. World Aquac. Soc. 36(1), 76-86. https://doi.org/https://doi.org/10.1111/j.1749-7345.2005.tb00133.x.

Nimrat, S., Siriboonlamom, S., Zhang, S., Xu, Y., Vuthiphandchai, V., 2006. Chilled storage of white shrimp (*Litopenaeus vannamei*) spermatophores. Aquaculture 261(3), 944-951. https://doi.org/https://doi.org/10.1016/j.aquaculture.2006.08.018.

Noor-Hidayati, A.B., Shahreza, M.S., Munafi, A.B.A.-., Ikhwanuddin, M., 2014. Sperm quality assessment of Banana Shrimp *Fenneropenaeus merguiensis* (De Man, 1888) from ultraviolet irradiation for initial development of gynogenesis application. J. Fish. Aquat. Sci. 9(4), 187-196. https://doi.org/https://dx.doi.org/10.3923/jfas.2014.187.196.

Noorbaiduri, S., Abol-Munafi, A.B., Ikhwanuddin, M., 2014. Acrosome reaction stage of sperm from mud crab, *Scylla olivacea* (Herbst, 1796): Mating in wild and in captivity. J. Fish. Aquat. Sci. 9(4), 237-244. https://doi.org/https://dx.doi.org/10.3923/jfas.2014.237.244.

Ogier de Baulny, B., Labbé, C., Maisse, G., 1999. Membrane integrity, mitochondrial activity, ATP content, and motility of the European Catfish (*Silurus glanis*) testicular spermatozoa after freezing with different cryoprotectants. Cryobiology 39(2), 177-184. https://doi.org/https://doi.org/10.1006/cryo.1999.2200.

Okada, A., Igarashi, H., Kuroda, M., Terao, K., Yoshikawa, Y., Sankai, T., 2001. Cryopreservation-induced acrosomal vesiculation in live spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). Hum. Reprod. 16(10), 2139-2147. https://doi.org/http://dx.doi.org/10.1093/humrep/16.10.2139.

Panner Selvam, M.K., Agarwal, A., 2018. A systematic review on sperm DNA fragmentation in male factor infertility: Laboratory assessment. Arab J. Urol. 16(1), 65-76. https://doi.org/https://doi.org/10.1016/j.aju.2017.12.001.

Parinaud, J., Mieusset, R., Vieitez, G., Labal, B., Richoilley, G., 1993. Influence of sperm parameters on embryo quality. Fertil. Steril. 60(5), 888-892. https://doi.org/https://doi.org/10.1016/s0015-0282(16)56292-x.

Parinaud, J., Richoilley, G., Vieitez, G., Labal, B., Moutaffian, H., 1996. Relevance of acrosome function in the evaluation of semen *in vitro* fertilizing ability. Fertil. Steril. 63(3), 598-603. https://doi.org/https://doi.org/10.1016/S0015-0282(16)57432-9.

Park, C., Chapman, F.A., 2005. An extender solution for the short-term storage of Sturgeon semen. N. Am. J. Aquac. 67(1), 52-57. https://doi.org/https://doi.org/10.1577/FA03-068.1.

Parnes, S., Sagi, A., 2002. Intensification of redclaw crayfish *Cherax quadricarinatus* culture. I. Hatchery and nursery system. Aquac. Eng. 26(4), 251-262. https://doi.org/https://doi.org/10.1016/S0144-8609(02)00034-1.

Pascual, C., Valera, E., Re-Regis, C., Gaxiola, G., Sanchez, A., Ramos, L., Soto, L.A., Rosas, C., 1998. Effect of water temperature on reproductive tract condition of *Penaeus setiferus* adult males. J. World Aquac. Soc. 29(4), 477-484. https://doi.org/https://doi.org/10.1111/j.1749-7345.1998.tb00672.x.

Paul, C., Murray, A.A., Spears, N., Saunders, P.T.K., 2008. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. Reproduction 136(1), 73-84. https://doi.org/https://doi.org/10.1530/rep-08-0036.

Pawar, R., Mohiuddin, M.I., 2000. Artificial insemination in penaeid shrimps. Indian J. Fish. 27, : 19-25.

Pegg, D.E., 2007. Principles of cryopreservation, in: Day, J.G., Stacey, G.N. (Eds.), Cryopreservation and freeze-drying protocols. Humana Press, Totowa, NJ, pp. 39-57.

Peixoto, S., Cavalli, R.O., Krummenauer, D., Wasielesky, W., D'Incao, F., 2004a. Influence of artificial insemination on the reproductive performance of *Farfantepenaeus paulensis* in conventional and unisex maturation systems. Aquaculture 230(1), 197-204. https://doi.org/https://doi.org/10.1016/S0044-8486(03)00431-9.

Peixoto, S., Cavalli, R.O., Wasielesky, W., D'Incao, F., Krummenauer, D., Milach, Â.M., 2004b. Effects of age and size on reproductive performance of captive *Farfantepenaeus paulensis* broodstock. Aquaculture 238(1), 173-182. https://doi.org/https://doi.org/10.1016/j.aquaculture.2004.04.024.

Peña, A.I., Quintela, L.A., Herradón, P.G., 1998. Viability assessment of dog spermatozoa using flow cytometry. Theriogenology 50(8), 1211-1220. https://doi.org/https://doi.org/10.1016/S0093-691X(98)00221-0.

Peña, S.T., Jr., Gummow, B., Parker, A.J., Paris, D.B.B.P., 2017. Revisiting summer infertility in the pig: could heat stress-induced sperm DNA damage negatively affect early embryo development? Anim. Prod. Sci. 57(10), 1975-1983. https://doi.org/https://doi.org/10.1071/AN16079.

Peña, S.T., Jr., Gummow, B., Parker, A.J., Paris, D.B.B.P., 2019a. Antioxidant supplementation mitigates DNA damage in boar (*Sus scrofa domesticus*) spermatozoa induced by tropical summer. *PLoS One* 14(4), e0216143. https://doi.org/https://doi.org/10.1371/journal.pone.0216143.

Peña, S.T., Jr., Stone, F., Gummow, B., Parker, A.J., Paris, D.B.B.P., 2019b. Tropical summer induces DNA fragmentation in boar spermatozoa: implications for evaluating seasonal infertility. Reprod. Fertil. Dev. 31(3), 590-601. https://doi.org/https://doi.org/10.1071/RD18159.

Pérez-Cerezales, S., Martínez-Páramo, S., Cabrita, E., Martínez-Pastor, F., de Paz, P., Herráez, M.P., 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71(4), 605-613. https://doi.org/https://doi.org/10.1016/j.theriogenology.2008.09.057.

Pérez-Rodríguez, J.C., Gómez-Gutiérrez, J., López-Greco, L.S., Cortés-Jacinto, E., 2019. Spermatophore production and sperm quality of the river prawn *Macrobrachium*

americanum Spence Bate, 1868 fed with different diets. Aquac. Res. 0(0), 1-13. https://doi.org/https://doi.org/10.1111/are.14265.

Perez-Velazquez, M., Bray, W.A., Lawrence, A.L., Gatlin, D.M., Gonzalez-Felix, M.L., 2001. Effect of temperature on sperm quality of captive *Litopenaeus vannamei* broodstock. Aquaculture 198(3), 209-218. https://doi.org/https://doi.org/10.1016/S0044-8486(01)00510-5.

Poljaroen, J., Vanichviriyakit, R., Tinikul, Y., Phoungpetchara, I., Linthong, V., Weerachatyanukul, W., Sobhon, P., 2010. Spermatogenesis and distinctive mature sperm in the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879). Zool. Anz. 249(2), 81-94. https://doi.org/https://doi.org/10.1016/j.jcz.2010.03.002.

Pongtippatee, P., Vanichviriyakit, R., Chavadej, J., Plodpai, P., Pratoomchart, B., Sobhon, P., Withyachumnarnkul, B., 2007. Acrosome reaction in the sperm of the black tiger shrimp *Penaeus monodon* (Decapoda, Penaeidae). Aquac. Res. 38(15), 1635-1644. https://doi.org/https://doi.org/10.1111/j.1365-2109.2007.01824.x.

Prasad, G.L.V., Naik, B.R., Ko, J.E., Nagaraju, G.P., 2014. Effects of naloxone, serotonin, and dopamine on reproduction of the freshwater crab *Barytelphusa guerini*. J. Exp. Zool. A. Ecol. Genet. Physiol. 321(3), 173-182. https://doi.org/https://doi.org/10.1002/jez.1847.

Qiu, J., Wang, W.-N., Wang, L.-j., Liu, Y.-F., Wang, A.-L., 2011. Oxidative stress, DNA damage and osmolality in the Pacific white shrimp, *Litopenaeus vannamei* exposed to acute low temperature stress. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 154(1), 36-41. https://doi.org/https://doi.org/10.1016/j.cbpc.2011.02.007.

Ravi, R., Manisseri, M.K., Sanil, N.K., 2014. Structure of the male reproductive system of the blue swimmer crab *Portunus pelagicus* (Decapoda: Portunidae). Acta Zool. 95(2), 176-185. https://doi.org/https://doi.org/10.1111/azo.12017.

Reers, M., Smiley, S.T., Mottola-Hartshorn, C., Chen, A., Lin, M., Chen, L.B., 1995. Mitochondrial membrane potential monitored by JC-1 dye, in: Methods in Enzymology. Academic Press, pp. 406-417.

Reynolds, J.D., 2002. Growth and Reproduction, in: Holdich, D.M. (Ed.), Biology of Freshwater Crayfish. Blackwell Science, Oxford, pp. 152–191.

Ron-El, R., Nachum, H., Herman, A., Golan, A., Caspi, E., Soffer, Y., 1991. Delayed fertilization and poor embryonic development associated with impaired semen quality. Fertil Steril. 55(2), 338-344. https://doi.org/https://doi.org/10.1016/S0015-0282(16)54127-2.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234(1), 1-28. https://doi.org/https://doi.org/10.1016/j.aquaculture.2003.12.006.

Rurangwa, E., Roelants, I., Huyskens, G., Ebrahimi, M., Kime, D.E., Ollevier, F., 1998. The minimum effective spermatozoa : egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilization ability in *Clarias gariepinus*. J. Fish. Biol. 53(2), 402-413. https://doi.org/https://doi.org/10.1111/j.1095-8649.1998.tb00989.x.

Sahtout, A.H., Hassan, M.D., Shariff, M., 2001. DNA fragmentation, an indicator of apoptosis, in cultured black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus (WSSV). Dis. Aquat. Org. 44(2), 155-159. https://doi.org/https://doi.org/10.3354/dao044155.

Samuel, M.J., Kannupandi, T., Soundarapandian, P., 1999. Nutritional effects on male reproductive performance in the freshwater prawn *Macrobrachium malcolmsonii* (H. Milne Edwards). Aquaculture 172(3), 327-333. https://doi.org/https://doi.org/10.1016/S0044-8486(98)00500-6.

Sandifer, P.A., Lawrence, A.L., Harris, S.G., Chamberlain, G.W., Stokes, A.D., Bray, W.A., 1984. Electrical stimulation of spermatophore expulsion in marine shrimp, *Penaeus* spp. Aquaculture 41(2), 181-187. https://doi.org/https://doi.org/10.1016/0044-8486(84)90096-6.

Sandifer, P.A., Lynn, J.W., 1980. Artificial insemination of caridean shrimp, in: W H Clark, J., Adams, T.S. (Eds.), Advances in Invertibrate Reproduction. Elsevier Press, North Holland, pp. 271-288.

Sandifer, P.A., Smith, T.I.J., 1979. A method for artificial insemiation *Macrobrachium* prawns and its potential use in inheritance and hybridization studies. J. World Aquac. Soc. 10(1-4), 403-418. https://doi.org/https://doi.org/10.1111/j.1749-7345.1979.tb00036.x.

Sarker, M.M., Islam, M.S., Uehara, T., 2009. Artificial insemination and early embryonic development of the mangrove crab *Perisesarma bidens* (De Haan) (Crustacea: Brachyura). Zool. Stud. 48(5), 607-618.

Sarojini, R., Nagabhushanam, R., Fingerman, M., 1993. *In vivo* evaluation of 5-hydroxytryptamine stimulation of the testes in the fiddler crab, *Uca pugilator*: A presumed action on the neuroendocrine system. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 106(2), 321-325. https://doi.org/https://doi.org/10.1016/0742-8413(93)90140-G.

Sato, T., Yoseda, K., Abe, O., Shibuno, T., 2008. Male maturity, number of sperm, and spermatophore size relationships in the coconut crab *Birgus Latro* on Hatoma Island, Southern Japan. J. Crust. Biol. 28(4), 663-668. https://doi.org/https://doi.org/10.1651/07-2966.1.

Sellos, D., Legal, Y., 1981. Changes in basic nuclear proteins during sperm maturation in *Palaemon serratus* (Crustacea natantia). Cell Death Differ. 10(2), 69-77. https://doi.org/https://doi.org/10.1016/0045-6039(81)90014-2.

Selvakumar, N., Krishnamoorthy, D., Beulah, C.D., Natesan, M., 2018. Retrieval and cryopreservation of sperm in spermatophores from cadaveric Indian white shrimp, *Fenneropenaeus indicus* (H. Milne Edwards, 1837). Anim. Reprod. Sci 192, 185-192. https://doi.org/https://doi.org/10.1016/j.anireprosci.2018.03.008.

Shu, L., Zhang, X., 2017. Shrimp miR-12 Suppresses White Spot Syndrome Virus infection by synchronously triggering antiviral phagocytosis and apoptosis pathways. Front. immunol. 8(855), 1-13. https://doi.org/https://doi.org/10.3389/fimmu.2017.00855.

Shuai, Z., Dong-fa, Z., Chun-lin, W., Liang-yi, X., 2007. Preservation of spermatozoa of blue crab *Portunus trituberculatus*. Oceanography 31(7), 37-42.

Siangcham, T., Tinikul, Y., Poljaroen, J., Sroyraya, M., Changklungmoa, N., Phoungpetchara, I., Kankuan, W., Sumpownon, C., Wanichanon, C., Hanna, P.J., Sobhon, P., 2013. The effects of serotonin, dopamine, gonadotropin-releasing hormones, and corazonin, on the androgenic gland of the giant freshwater prawn, *Macrobrachium rosenbergii*. Gen. Comp. Endocrinol. 193, : 10-18. https://doi.org/https://doi.org/10.1016/j.ygcen.2013.06.028.

Sieme, H., Oldenhof, H., Wolkers, W.F., 2016. Mode of action of cryoprotectants for sperm preservation. Anim. Reprod. Sci. 169, : 2-5. https://doi.org/https://doi.org/10.1016/j.anireprosci.2016.02.004.

Silva, E.F., Batista, A.M., Silva, S.V., Calazans, N., Castelo Branco, T., Guerra, M.M.P., Peixoto, S., 2015. Use of fluorescent microscopy for sperm quality of penaeids. J. Crust. Biol. 35(1), 26-29. https://doi.org/https://doi.org/10.1163/1937240X-00002295.

Simon, L., Carrell, D.T., 2013. Sperm DNA Damage Measured by Comet Assay. In: Carrell D., Aston K. (eds) Spermatogenesis. Methods in Molecular Biology (Methods and Protocols). Methods Mol. Biol. 927, : 137-146. https://doi.org/https://doi.org/10.1007/978-1-62703-038-0_13.

Simon, L., Murphy, K., Shamsi, M.B., Liu, L., Emery, B., Aston, K.I., Hotaling, J., Carrell, D.T., 2014. Paternal influence of sperm DNA integrity on early embryonic development. Hum. Reprod. 29(11), 2402-2412. https://doi.org/https://doi.org/10.1093/humrep/deu228.

Stewart, M.J., Stewart, P., Soonklang, N., Linthong, V., Hanna, P.J., Duan, W., Sobhon, P., 2010. Spermatogenesis in the blue swimming crab, *Portunus pelagicus*, and evidence for histones in mature sperm nuclei. Tissue Cell 42(3), 137-150. https://doi.org/https://doi.org/10.1016/j.tice.2010.03.002.

Subramoniam, T., 1993. Spermatophores and sperm transfer in marine crustaceans, in: Blaxter, J.H.S., Southward, A.J. (Eds.), Advances in Marine Biology. Academic Press, Elsevier Inc., pp. 129-214.

Subramoniam, T., 2017a. Chapter 11 - Spermatogenesis, in: Subramoniam, T. (Ed.), Sexual Biology and Reproduction in Crustaceans. Academic Press, Elsevier Inc., pp. 293-324.

Subramoniam, T., 2017b. Chapter 14 - Fertilization, in: Subramoniam, T. (Ed.), Sexual Biology and Reproduction in Crustaceans. Academic Press, Elsevier Inc., pp. 391-418.

Subramoniam, T., 2017c. Spermatophore and Sperm Transfer Mechanisms, in: Sexual Biology and Reproduction in Crustaceans. Academic Press, Elsevier Inc., pp. 325-368.

Talbot, P., Beach, D., 1989. Role of the vas deferens in the formation of the spermatophore of the crayfish (*Cherax*). J. Crust. Biol. 9(1), 9-24. https://doi.org/https://doi.org/10.1163/193724089X00160.

Talbot, P., Chacon, R.S., 1981. A triple-stain technique for evaluating normal acrosome reactions of human sperm. J. Exp. Zool. 215(2), 201-208. https://doi.org/https://doi.org/10.1002/jez.1402150210.

Talbot, P., Chanmanon, P., 1980. Morphological features of the acrosome reaction of lobster (*Homarus*) sperm and the role of the reaction in generating forward sperm movement. J. Ultrastruct. Res. 70(3), 287-297. https://doi.org/https://doi.org/10.1016/S0022-5320(80)80012-8.

Talbot, P., Thaler, C., Wilson, P., 1986. Artificial insemination of the American lobster (*Homarus*). Gamete Res. 14(1), 25-31. https://doi.org/https://doi.org/10.1002/mrd.1120140104.

Tandara, M., Bajić, A., Tandara, L., Bilić-Zulle, L., Šunj, M., Kozina, V., Goluža, T., Jukić, M., 2014. Sperm DNA integrity testing: big halo is a good predictor of embryo quality and pregnancy after conventional IVF. Andrology 2(5), 678-686. https://doi.org/http://dx.doi.org/10.1111/j.2047-2927.2014.00234.x.

Tello-Mora, P., Hernández-Cadena, L., Pedraza, J., López-Bayghen, E., Quintanilla-Vega, B., 2018. Acrosome reaction and chromatin integrity as additional parameters of semen analysis to predict fertilization and blastocyst rates. Reprod. Biol. 16(1), 102. https://doi.org/https://doi.org/10.1186/s12958-018-0408-0.

Tereza, C.V.G., Halcrow, K., 1988. Influence of some external factors on the acrosome reaction in the spermatozoa of *Homarus americanus* Milne-Edwards, 1837. J. Crust. Biol. 8(3), 317-321. https://doi.org/https://doi.org/10.1163/193724088X00189.

Tropea, C., Hermida, G.N., López Greco, L.S., 2011. Effects of androgenic gland ablation on growth and reproductive parameters of *Cherax quadricarinatus* males (Parastacidae, Decapoda). Gen. Comp. Endocrinol. 174(2), 211-218. https://doi.org/https://doi.org/10.1016/j.ygcen.2011.08.023.

Tsai, K.L., Talbot, P., 1993. Video microscopic analysis of ionophore induced acrosome reactions of lobster (*Homarus americanus)* sperm. Mol. Reprod. Dev. 36(4), 454-461. https://doi.org/https://doi.org/10.1002/mrd.1080360408.

Uberti, M.F., Vieira, F.d.N., Salência, H.R., Vieira, G.d.S., Vinatea, L.A., 2014. Assessment of viability of sperm cells of *Litopenaeus vannamei* on cryopreservation. Braz. Arch. Biol. Technol. 57(3), 374-380. https://doi.org/https://doi.org/10.1590/S1516-89132014005000013.

Valentina-Claudet, P., Narasimman, S., Natesan, M., 2016. Effect of cryoprotectants and cooling rates on fertility potential of sperm in the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man). Anim. Reprod. Sci. 171, : 49-57. https://doi.org/https://doi.org/10.1016/j.anireprosci.2016.05.013.

Van den Berghe, F., Paris, M.C.J., Briggs, M.B., Farstad, W.K., Paris, D.B.B.P., 2018. A two-step dilution tris-egg yolk extender containing Equex STM significantly improves sperm cryopreservation in the African wild dog (*Lycaon pictus*). Cryobiology 80, 18-25. https://doi.org/https://doi.org/10.1016/j.cryobiol.2017.12.095.

Van Harreveld, A., 1936. A physiological solution for freshwater crustaceans. Proc. Soc. Exp. Biol. Med. 34(4), 428-432. https://doi.org/https://doi.org/10.3181%2F00379727-34-8647C.

Vanichviriyakit, R., Kruevaisayawan, H., Weerachatyanukul, W., Tawipreeda, P., Withyachumnarnkul, B., Pratoomchat, B., Chavadej, J., Sobhon, P., 2004. Molecular modification of *Penaeus monodon* sperm in female thelycum and its consequent responses. Mol. Reprod. Dev. 69(3), 356-363. https://doi.org/https://doi.org/10.1002/mrd.20138.

Vázquez-Islas, G., Racotta, I.S., Robles-Romo, A., Campos-Ramos, R., 2013. Energy balance of spermatophores and sperm viability during the molt cycle in intact and bilaterally eyestalk ablated male Pacific white shrimp *Litopenaeus vannamei*. Aquaculture 414-415, : 1-8. https://doi.org/https://doi.org/10.1016/j.aquaculture.2013.07.020.

Vazquez, F.J., López-Greco, L.S., 2010. Differentiation of the sexual characters and onset of maturity in the males of the "red claw" crayfish, *Cherax quadricarinatus* (Von Martens, 1898) (Astacidea, Astacida, Parastacidae). Anim. Biol. 60(2), 195-208. https://doi.org/https://doi.org/10.1163/157075610X491734.

Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P., Kopf, G.S., 1995. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway.

Development 121(4), 1139-1150. https://doi.org/https://doi.org/10.1242/dev.121.4.1139.

Vuthiphandchai, V., Nimrat, S., Kotcharat, S., Bart, A.N., 2007. Development of a cryopreservation protocol for long-term storage of black tiger shrimp (*Penaeus monodon*) spermatophores. Theriogenology 68(8), 1192-1199. https://doi.org/https://doi.org/10.1016/j.theriogenology.2007.08.024.

Wagner, H., Cheng, J.W., Ko, E.Y., 2018. Role of reactive oxygen species in male infertility: An updated review of literature. Arab J. Urol. 16(1), 35-43. https://doi.org/https://doi.org/10.1016/j.aju.2017.11.001.

Wang, L., Xu, T., Lei, W.-w., Liu, D.-m., Li, Y.-j., Xuan, R.-j., Ma, J.-j., 2011. Cadmium-induced oxidative stress and apoptotic changes in the testis of freshwater crab, *Sinopotamon henanense*. *PLoS One*. 6(11), e27853. https://doi.org/https://doi.org/10.1371/journal.pone.0027853.

Wang, Q., Misamore, M., Jiang, C.Q., Browdy, C.L., 1995. Egg water induced reaction and biostain assay of sperm from marine shrimp *Penaeus vannamei*: Dietary effects on sperm quality. J. World Aquac. Soc. 26(3), 261-271. https://doi.org/https://doi.org/10.1111/j.1749-7345.1995.tb00254.x.

Wang, W.-N., Zhou, J., Wang, P., Tian, T.-T., Zheng, Y., Liu, Y., Mai, W.-j., Wang, A.-L., 2009. Oxidative stress, DNA damage and antioxidant enzyme gene expression in the Pacific white shrimp, *Litopenaeus vannamei* when exposed to acute pH stress. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 150(4), 428-435. https://doi.org/https://doi.org/10.1016/j.cbpc.2009.06.010.

Wang, Y.-L., Sun, W.-J., He, L., Li, Q., Wang, Q., 2015. Morphological alterations of all stages of spermatogenesis and acrosome reaction in Chinese mitten crab *Eriocheir sinensis*. Cell Tissue Res. 360(2), 401-412. https://doi.org/https://doi.org/10.1007/s00441-014-2092-5.

Wilson, G., 2009. The phylogenetic position of the Isopoda in the Peracarida (Crustacea: Malacostraca). Arthropod Syst. Phylogeny 67(2), 159-198.

Wowk, B., 2007. How cryoprotectants work, Cryonics. Alcor Life Extension Foundation, 7895 East Acoma Drive, Suite 110, Scottsdale, Arizona 85260, pp. 3-7.

Wowk, B., 2010. Thermodynamic aspects of vitrification. Cryobiology 60(1), 11-22. https://doi.org/https://doi.org/10.1016/j.cryobiol.2009.05.007.

XingHong, X., BinLun, Y., JiaTao, X., GuoCheng, X., YinFang, P., JianHe, X., 2010. Cryopreservation of spermatozoa in Japanese swimming crab *Charybdis japonica*. Fish. Sci. 29(10), 601-604.

Xu, X.H., Xu, X.H., Yan, B.L., Xu, X.H., Yan, B.L., Peng, D.S., 2014. Comparative study on the acrosin activity in sperm of the marine crab *Charybdis japonica* (A. Milne-Edwards, 1861) (Brachyura, Portunidae) under different environmental conditions. Crustaceana 87(13), 1473-1485. https://doi.org/https://doi.org/10.1163/15685403-00003369.

Yamaguchi, S., Celino, F.T., Ito, A., Agusa, T., Tanabe, S., Tuyen, B.C., Miura, C., Miura, T., 2008. Effects of arsenic on gonadal development in freshwater crab, *Somanniathelphusa pax*, in Vietnam and *Geothelphusa dehaani* in Japan. Ecotoxicology 17(8), 772-780. https://doi.org/https://doi.org/10.1007/s10646-008-0228-7.

Yang, G., Kille, P., Ford, A.T., 2008. Infertility in a marine crustacean: Have we been ignoring pollution impacts on male invertebrates? Aquat. Toxicol. 88(1), 81-87. https://doi.org/https://doi.org/10.1016/j.aquatox.2008.03.008.

Yasuzumi, G., Kaye, G.I., Pappas, G.D., Yamamoto, H., Tsubo, I., 1961. Nuclear and cytoplasmic differentiation in developing sperm of the crayfish, *Cambaroides japonicus*. Z. Zellforsch Mikrosk. Anat. 53(2), 141-158. https://doi.org/https://doi.org/10.1007/BF00339438.

Yasuzumi, G., Lee, K.J., 1966. Spermatogenesis in animals as revealed by electron microscopy XVI. The microtubular structure and sites of thiamine pyrophosphatase activity in premature sperm of the Japanese crayfish). Z. Zellforsch Mikrosk. Anat. 73(3), 384-404. https://doi.org/https://doi.org/10.1007/BF00342583.

Yazicioglu, B., Hamr, P., Kozák, P., Kouba, A., Niksirat, H., 2016. Fine structure of the spermatozoon in three species of Cambaridae (Arthropoda: Crustacea: Decapoda) *Cambarus robustus, Orconectes propinquus* and *Orconectes rusticus*: a comparative biometrical study. PeerJ 4, : e2363-e2363. https://doi.org/https://doi.org/10.7717/peerj.2363.

Yazicioglu, B., Kouba, A., Kozák, P., Niksirat, H., 2018. Post-mating spermatophore storage strategies in two species of crayfish: implications for broodstock management. Animal 12(3), 554-558. https://doi.org/https://doi.org/10.1017/S1751731117001744.

Yildiz, C., Ottaviani, P., Law, N., Ayearst, R., Liu, L., McKerlie, C., 2007. Effects of cryopreservation on sperm quality, nuclear DNA integrity, in vitro fertilization, and in vitro embryo development in the mouse. Reproduction. 133(3), 585-595. https://doi.org/https://doi.org/10.1530/rep-06-0256.

Zandemami, M., Qujeq, D., Akhondi, M.M., Kamali, K., Raygani, M., Lakpour, N., Shiraz, E.S., Sadeghi, M.R., 2012. Correlation of CMA3 staining with sperm quality and protamine deficiency. Lab. Med. 43(6), 262-267. https://doi.org/https://doi.org/10.1309/LMB42F9QXYKFLJNG.

Zara, F.J., Toyama, M.H., Caetano, F.H., López-Greco, L.S., 2012. Spermatogenesis, spermatophore, and seminal fluid production in the adult blue crab *Callinestes danae* (Portunidae). J. Crust. Biol. 32(2), 249-262. https://doi.org/https://doi.org/10.1163/193724011X615479.

Zhang, Z., Cheng, H., Wang, Y., Wang, S., Xie, F., Li, S., 2010. Acrosome reaction of sperm in the mud crab *Scylla serrata* as a sensitive toxicity test for metal exposures. Arch. Environ. Contam. Toxicol. 58(1), 96-104. https://doi.org/https://doi.org/10.1007/s00244-009-9326-7.

Zhao, D., Zhang, X., Li, X., Ru, S., Wang, Y., Yin, J., Liu, D., 2019. Oxidative damage induced by copper in testis of the red swamp crayfish *Procambarus clarkii* and its underlying mechanisms. Aquat. Toxicol. 207, : 120-131. https://doi.org/https://doi.org/10.1016/j.aquatox.2018.12.006.

Zheng, W.-W., Song, G., Wang, Q.-L., Liu, S.-W., Zhu, X.-L., Deng, S.-M., Zhong, A., Tan, Y.-M., Tan, Y., 2018. Sperm DNA damage has a negative effect on early embryonic development following *in vitro* fertilization. Asian J. Androl. 20(1), 75-79. https://doi.org/https://dx.doi.org/10.4103%2Faja.aja_19_17.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., Vilella, S., 2003. Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology 47(3), 227-235. https://doi.org/https://doi.org/10.1016/j.cryobiol.2003.10.002.

Chapter 3. Use of optimised conventional and advanced sperm quality diagnostic tools to establish reproductive data in wild-caught redclaw crayfish (*Cherax quadricarinatus*)

3.1. Abstract

The Australian redclaw crayfish has great potential for aquaculture intensification and global market expansion. However, traditional production methods, subfertility, and high embryo mortality could curtail industry growth. Successful embryonic and post-embryonic development also depend on the paternal genetic contribution. Direct assessment of sperm quality can potentially determine the status of male fertility in decapod crustaceans. This study evaluated redclaw crayfish sperm quality using optimised conventional and advanced functional tools. Sexually mature male redclaw (n = 33) were collected from Ross River Dam, North Queensland, Australia and subjected to electroejaculation to yield spermatophores for sperm quality assessment. Sperm concentration and count (haemocytometer), morphology (phase contrast), viability (Hoechst 33342/PI), DNA fragmentation (TUNEL), and total potential fertile sperm concentration (TPFSC) were determined. Spermatozoa had an elliptical shape of varying sizes and a presumptive tail-like structure. The mean ± SEM sperm viability, DNA fragmentation, concentration, and TPFSC were 65.2 ± 3.9 %, 17.2 ± 2.5 %, 42.5 x 10^4 ± 5.1 x 10^4 cells/mL, and 23.6 x 10^4 ± 3.4 x 10^4 cells/mL, respectively. Sperm concentration ($p = 0.001$) and TPFSC ($p < 0.001$) were positively associated with spermatophore weight, whereas sperm DNA fragmentation ($p = 0.022$) was inversely associated, but not sperm viability ($p = 0.188$). Sperm viability was also negatively associated with body mass ($p = 0.010$). In conclusion, this study reported the first

quantitative data on sperm morphometry and functional traits, validated species-specific diagnostic tools for sperm quality assessment, and provided information on the relationships between body and spermatophore weights and sperm quality parameters. Our approach may inform the selection of fertile male broodstock and enhanced strategies for spermatozoa harvest for selective breeding programs for commercial redclaw aquaculture.

Keywords: Fertility marker, crustacean, DNA fragmentation, electroejaculation, spermatophore, sperm viability

3.2. Introduction

Redclaw crayfish (*Cherax quadricarinatus*) are increasingly recognised for their farming and intensification potential in aquaculture (Rigg et al., 2020; Haubrock et al., 2021). It is a non-burrowing, large, hardy and fast-growing freshwater crayfish native to Northern Australia (Jones et al., 1996; Jones, 2011). Redclaw are easy to breed, can withstand high stocking rates, and can tolerate a wide range of environmental conditions, making them an attractive commodity in aquaculture (Jones, 1990; Jones et al., 1996; Jones, 2000). In 2017, global production of redclaw crayfish reached 239 tonnes equating to US$ 2.4 million (FAO-FIGIS, 2019), a volume that is projected to continue to expand (Irvin et al., 2018). Despite the economic potential, the redclaw farming industry faces multiple challenges, including insufficient seedstock supply, a limited production season, and an economically inefficient and small scale of production (Jones et al., 1996; Rigg et al., 2020). The supply of adult redclaw to the crustacean market is hindered by insufficient production of juveniles from hatcheries for large-scale farming (Medley et al., 1994; Jones, 2000, 2011; Rigg et al., 2020). Seasonal factors in the traditional hatchery-nursery system in earthen ponds limit the

production of juveniles mainly to the warmer months of the year, with low rates of survival (typically 5 – 10 %). Economic returns are also detrimentally affected by the inability of producers to feasibly monitor and manage the quality, age, and growth of redclaw in earthen pond systems, which contributes to variable juvenile sizes and high rates of cannibalism (Jones, 1995; Masser and Rouse, 1997; Stevenson et al., 2013; Irvin et al., 2018; Rigg et al., 2020).

For more than four decades, redclaw hatcheries have developed intensified breeding systems in an attempt to improve juvenile yields and reduce variation in size (Jones, 1995; Parnes and Sagi, 2002; Jones and Valverde, 2020). While such systems offer clear advantages, several problems have been encountered, including a high proportion of unhatched embryos, hatched embryos with deformities, and mortalities that limit the yield of early juveniles (Jones and Valverde, 2020; Elliot, pers. comm.). While significant advances have been made in broodstock handling and husbandry techniques in modern hatchery and breeding systems (Jones, 1995; Parnes and Sagi, 2002; Jones and Valverde, 2020; Rigg et al., 2020), there has been limited focus on the impact of broodstock fertility and reproductive efficiency on juvenile production. The fertility of male broodstock is likely to play a crucial role in the productivity of intensive hatcheries.

In aquaculture, the reproductive efficiency of farmed crustaceans has primarily focussed on egg and embryo, rather than sperm quality. However, the competence of eggs and spermatozoa affects fertilisation success, embryonic and larval development, and survival (Bobe and Labbé, 2010). As such, the assessment of sperm quality should be a key consideration when evaluating and optimising reproductive productivity within hatcheries (Rurangwa et al., 2004; Marc et al., 2021). In all species, subfertile males can limit the

production of offspring (Janny and Menezo, 1994; Rurangwa et al., 2004; Riesco et al., 2019). For example, the degree of sperm DNA damage in mammals is associated with the extent of apoptosis and cellular damage within embryos, resulting in delayed or arrested embryonic development, low rates of blastocyst formation and pregnancy and high rates of abortion (Simon et al., 2014; Peña et al., 2017; Zheng et al., 2018), and it does not clearly predict fertilisation rate (Kumar et al., 2013). Quantifying sperm DNA integrity may facilitate the identification and removal of potentially infertile or subfertile male broodstock, thereby improving productivity and decreasing operational costs in crustacean production hatchery systems (Feng, 2018; Aquino et al., 2022).

Evaluation of male fertility through sperm quality assessment has been used to detect deleterious effects caused by suboptimal health, nutrition, husbandry, genetics or other factors in aquaculture species (Lewis and Ford, 2012; Cabrita et al., 2014; Harlıoğlu and Farhadi, 2017; Harlıoğlu et al., 2018; Aquino et al., 2022). Measurements using traditional sperm quality assays (including evaluation of motility, morphology, and concentration) have been used as a predictor of fertility; however, results are often variable, inaccurate, and unreliable (Graham et al., 1990; Gillan et al., 2005; Duangjai et al., 2023). In addition, traditional sperm quality assays may not be directly applicable to redclaw since spermatozoa are non-motile and non-flagellated (Beach and Talbot, 1987; Kouba et al., 2015; Subramoniam, 2017a, 2017b). Evaluating a single sperm quality biomarker is insufficient to provide robust reproductive data (Graham et al., 1980), however the combination of assessment of several traits can provide a better predictor of male fertility and fertilising capacity (Graham et al., 1990). The development of more advanced functional assays as an adjunct to traditional approaches has the potential to improve fertility prediction at the species level and would be invaluable to the industry (Graham et al., 1990; Cabrita et al., 2009; Aquino et al., 2022).

Fluorescent staining of plasma, acrosome, and mitochondrial membranes, and DNA, combined with fluorescent microscopy or flow cytometry, is increasingly used to evaluate sperm quality in aquaculture species (De Baulny et al., 1997; Lezcano et al., 2004; Gillan et al., 2005; Liu et al., 2007; Favret and Lynn, 2010; Qiu et al., 2011; Lewis and Ford, 2012; Erraud et al., 2018; Marc et al., 2021; Aquino et al., 2022). Moreover, flow cytometry facilitates rapid and accurate quantification of sperm quality on a larger scale than fluorescent microscopy (Lezcano et al., 2004; Gillan et al., 2005; Hossain et al., 2011). However, basic methods to evaluate sperm quality, including fluorescent biomarkers and flow cytometry, have not been described for redclaw crayfish. Therefore, this study aimed to use basic methods to evaluate sperm quality and develop fluorescent and flow cytometric techniques for quantifying sperm viability and DNA integrity as a predictor of male fertility in wild-caught redclaw crayfish.

3.3. Material and Methods

3.3.1. Experimental animals

Male redclaw crayfish were collected using baited funnel traps (Jones, 1990) along the Ross River Dam, Townsville, Queensland (19.4090° S, 146.7348° E) during summer (January – February 2022). Baited traps were set adjacent to riverbanks no deeper than two metres late in the afternoon and were collected the following morning. Sexually mature male redclaw (> 50 g) were transported to the Australian Crayfish Hatchery (ACH, Townsville, Queensland) recirculating facility for disinfection. Disinfection involved immersion in saltwater (30 ppt) for 20 min before placement in a clean tank with vigorous aeration (Sugiani et al., 2015). Animals were weighed and measured before being subjected to electroejaculation. The

Institutional Animal Ethics Committee deemed crustaceans exempt from animal ethics requirements, negating the need for ethics approval. This study conforms to ACH protocols for ethical animal treatment and industry best practices.

3.3.2. Spermatophore extraction and sperm preparation

Spermatophore collection via electroejaculation was performed using the protocol described by Jerry (2001), with some modifications. Body weight (BW), carapace length (CL) and width (CW), and total body length (TL) of sexually mature male redclaw (n = 33) at the intermoult stage were measured after being anaesthetised in chilled freshwater (15 $^{\circ}$C) for 10 min before electroejaculation. Gonopores were swabbed with 70 % ethanol to eliminate surface bacteria that could contaminate spermatophores during extrusion. Crayfish were held in a retainer tube with gonopores facing the operator (Kooda-Cisco and Talbot, 1983). A pair of electrodes connected to an AC variable transformer was set at the base of the abdominal keel before the fifth pereiopods. Electrical stimulation was applied at 36 volts, 0.01 A, and cycle frequency between 50 – 60 Hz with 1 – 2 sec intervals until spermatophores were expelled or a maximum of ten attempts were made. Extruded spermatophores were immersed in 1 mL Ca^{2+}- free saline (CFS) solution (370.1mM NaCl, 15.0 mM KCl, 8.6 mM H_3BO_3, 4.8 mM NaOH, and 20.0 mM $MgSO_4 \cdot 7H_2O$ at pH 7.4) immediately after extrusion and weighed (Leung-Trujillo and Lawrence, 1987). The use of CFS solution is a recommended media for sperm quality assessments because the absence of calcium prevents initiation of the acrosome reaction in spermatozoa and is widely used for other decapod crustaceans (Samuel et al., 1999; Vuthiphandchai et al., 2007; Xu et al., 2014; Feng et al., 2018).

To characterise its structure, the morphology of the spermatophore was studied using another group (n = 3) of sexually mature redclaw males. Briefly, spermatophores were bisected and suspended in CFS solution in two separate tubes. One tube was stained with 10 µg/mL Hoechst 33342 (H_{342}; Sigma-Aldrich Pty. Ltd., Macquarie Park NSW, Australia) for 40 min, protected from light and at room temperature (RT, 22 – 24 °C). Hoechst-stained spermatophores were examined and photographed under a fluorescent inverted microscope (Leica DMIL coupled with an Olympus DP74 camera/CoolLED pE-300 W fluorescent light source, Andover, UK), 24 h after electroejaculation. The second tube was observed and photographed under stereo (Olympus SZX7-ILLT, Tokyo, Japan, coupled with a Basler ace acA2440-75uc camera, Ahrensburg, Germany) and phase-contrast microscopes (Olympus BX53, Tokyo, Japan, coupled with a Basler aviator avA1000-gc camera, Ahrensburg, Germany), 96 h after electroejaculation.

To analyse fertility among the n = 33 males, a single-sperm cell suspension was prepared by dissecting the spermatophore into five approximately equal-sized segments and suspending these in CFS solution for 24 h, at RT. After 24 h, the liberation of spermatozoa into the CFS solution was maximised by gentle repeat-aspiration using a 1,000 µl pipette tip (10 x), after which the residual spermatophores were removed. The CFS-sperm cell solution was then used for subsequent assessment of sperm concentration and morphology, sperm viability and DNA fragmentation.

3.3.3. Sperm number, concentration, and morphology

The concentration and total number of spermatozoa in each spermatophore were manually counted at 100 X magnification using a Neubauer haemocytometer chamber (ProSciTech,

Townsville, Queensland) under phase-contrast microscopy (Olympus BX53, Tokyo, Japan; WHO, 2021), with minor modifications. Briefly, 10 µl of a single sperm cell suspension was placed into a haemocytometer chamber and permitted to settle for 3 min, prior to enumeration at 100 X final magnification. Since redclaw spermatozoa were sticky, glass slides and coverslips were washed with soap, dried, and cleaned thoroughly with 80 % ethanol between assessments. Sperm count per gram of spermatophore was calculated using the total suspended spermatozoa divided by the spermatophore weight. The total suspended spermatozoa were derived from the sperm concentration multiplied by the total volume of single-sperm cell suspension in the tube (1 mL).

For sperm morphology, a 10 µL aliquot of single-cell suspension was smeared on a glass slide according to World Health Organisation guidelines (WHO, 2021). Spermatozoa were analysed by phase contrast illumination at 400 X magnification (Olympus BX53, Tokyo, Japan, coupled with a Basler avA1000 - 100 gc camera; Basler AG, Ahrensburg, Germany). Micrographs of redclaw spermatozoa were captured using AndroVision® morphology and morphometry software (Version 1.1; Minitüb GmbH, Tiefenbach, Germany), and biometric traits were measured using ImageJ software (Version 1.53t) after calibration to a micrometre scale (Schindelin et al., 2012). Biometric traits were measured using ImageJ's freehand tool, including head length (L, the longest distance of an ellipse) and width (W, the widest distance perpendicular to head length). The head ratio (ellipticity), area, perimeter, surface area, and volume were calculated using the length and width data. To establish baseline morphology ranges, 300 spermatozoa from 20 randomly selected male redclaw were assessed. The formula below calculates different sperm morphology parameters (Marc et al., 2021).

Head ratio (ellipticity) = L/W

$$Head\ area = \pi * L/2 * W/2$$

$$Head\ perimeter = 2\pi*[\sqrt{(L^2 + W^2)}]/2$$

$$Head\ surface\ area = 4\pi*([W/2]^2 + [L - W] + [2\pi*(W/2)])$$

$$Head\ volume = (4/3*\pi)*(L/2)*(W/2)^2$$

3.3.4. Sperm viability assessment

Sperm viability was assessed by measuring the integrity of the plasma membrane through dual nuclear staining using a membrane-permeant stain, Hoechst 33342 (H_{342}; Sigma-Aldrich Pty. Ltd., Macquarie Park NSW, Australia), and a membrane-impermeant stain, propidium iodide (PI; Sigma-Aldrich Pty. Ltd., Macquarie Park NSW, Australia), following the protocol of Marc et al. (2021), with minor modifications. Samples from each male redclaw were adjusted to 1.0×10^5 spermatozoa/mL in CFS. Staining methods, flow cytometry gating, and compensation parameters were initially optimised using a representative sperm population created by pooling 7.0×10^4 spermatozoa from 7 redclaw samples. An unstained negative control (U_1), an H_{342} single-stained positive control (H_{342}^+), a fixed and permeabilised PI single-stained positive control (PI^+), and a fixed and permeabilised double-stained H_{342}/PI-positive control (H_{342}^+/PI^+) was prepared from the pooled sperm sample.

The H_{342}^+ control was prepared by incubating fresh (unfixed) pooled spermatozoa with 10 µg/mL H_{342} (40 min, RT, protected from light). For PI^+ single-stained and double-stained H_{342}^+/PI^+ positive controls, pooled spermatozoa samples were fixed with 4 % formaldehyde (dissolving paraformaldehyde in CFS solution; Sigma-Aldrich Pty. Ltd., Macquarie Park NSW, Australia) on a shaker (450 rpm) for 1 h at RT. Fixation was performed one hour after

generating the single-cell suspension. After fixation, samples were centrifuged (200 x g, 5 min), the supernatant removed, and resuspended cell pellets were washed twice with CFS solution. Fixed cells were permeabilised with 0.1 % Triton X-100 in 0.1 % sodium citrate solution (4 $^{\circ}$C, 2 min), then washed twice with CFS (200 x *g*, 5 min). The single-stained PI^+ control was prepared by resuspending the cell pellet in 10 µg/mL PI for 10 min at RT. For the double-stained H_{342}^+/PI^+ control, cells were resuspended and incubated at RT in 10 µg/mL H_{342} for 40 min, followed by 10 µg/mL PI for 10 min (RT, protected from light). This fixation and permeabilisation protocol disrupted plasma membrane in 93.3 ± 0.3 % (mean ± SEM) of spermatozoa.

The same conditions were used for H_{342} and PI staining of test samples; however, staining was performed on fresh (unfixed and unpermeabilised) spermatozoa. All samples were then washed twice with CFS solution and re-suspended to a final volume of 1 mL with 2 mM EDTA in CFS (pH 7.4) prior to flow cytometry analysis.

Staining specificity and efficiency were confirmed visually by fluorescence microscopy (Olympus BX53/CoolLED pE-300 W fluorescent microscope, Tokyo, Japan) at 100 X final magnification, using the blue (Ex/Em = 343/483) and red (Ex/Em = 536/617) channels, prior to flow cytometry. Sperm nuclei were stained blue by H_{342}, while the membrane-disrupted (dead) spermatozoa nuclei were stained pink/red by PI. Double-stained H_{342}^+/PI^+ spermatozoa were considered damaged/dead cells (Figures 3.1 a – b). Flow cytometry was performed two hours after generating the single-cell suspension, and the percentage of viable spermatozoa was determined for each test sample (Figure 3.2 g).

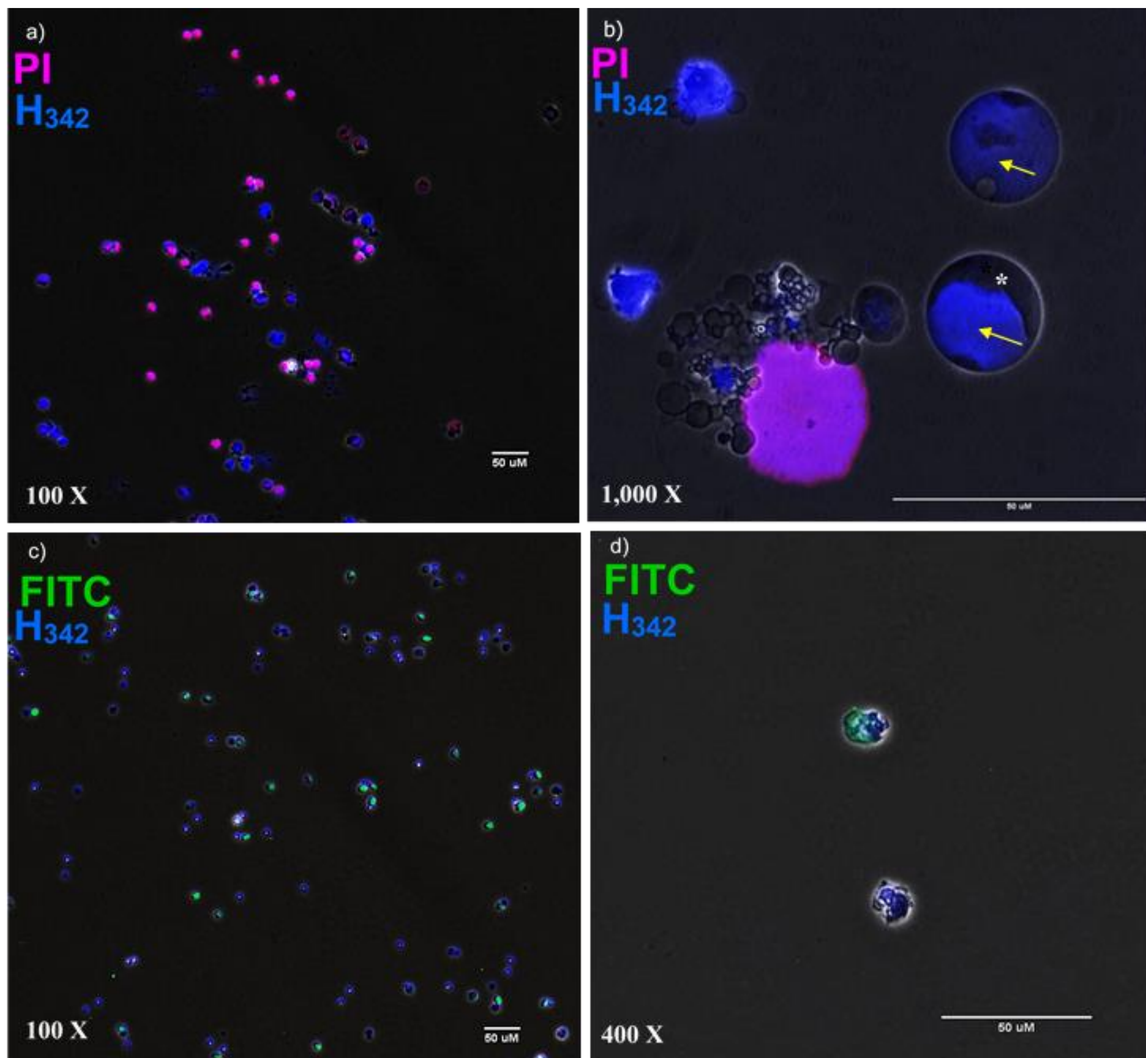
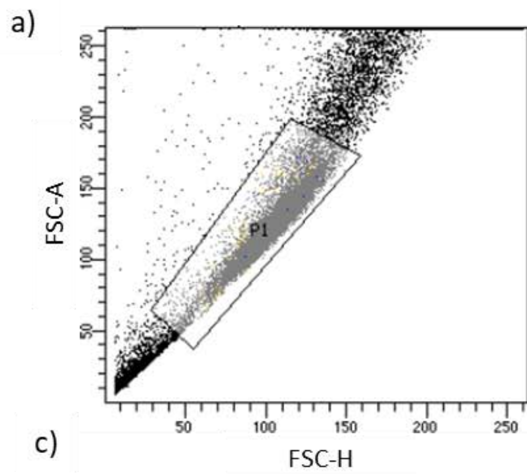
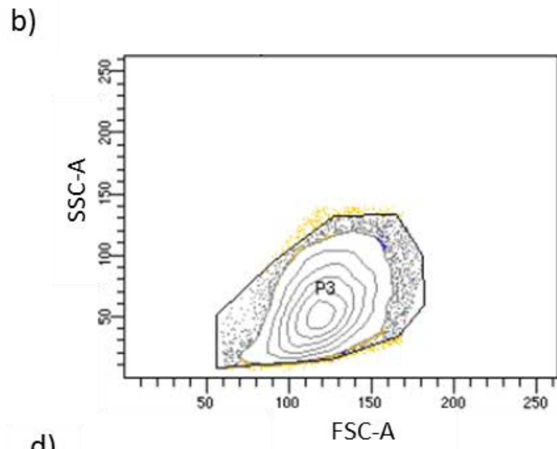


Figure 3.1. Validation of nuclear staining specificity of (a, b) viability and (c, d) DNA fragmentation assays for *C. quadricarinatus* displaying intact/viable (blue; H_{342}^{+}), membrane-damaged/dead (pink; PI^{+}/ H_{342}^{+}), or DNA-damaged (green; $FITC^{+}$/ H_{342}^{+}) spermatozoa. FITC, fluorescein isothiocyanate; H_{342}, Hoechst 33342; PI, propidium iodide; *, acrosome; yellow arrows - nuclear region; scale bar = 50 µm.

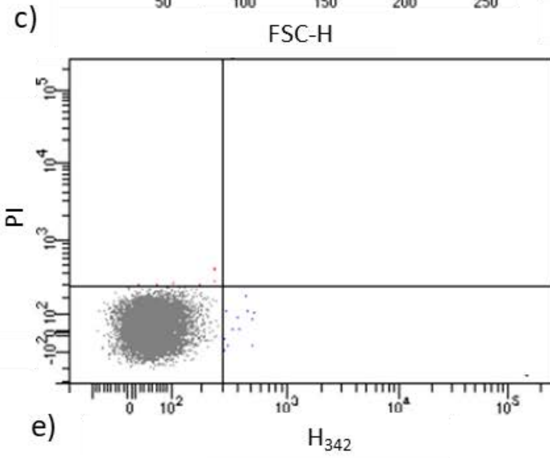
a)



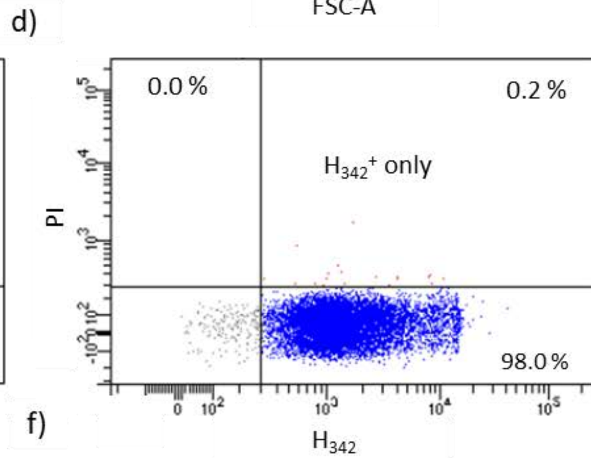
b)



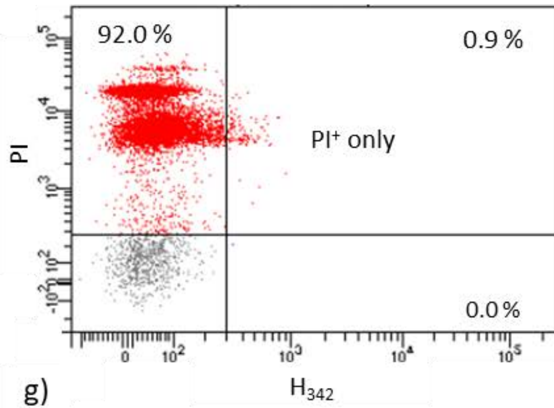
c)



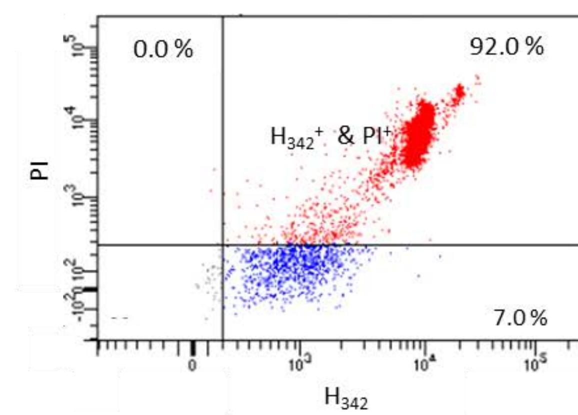
d)



e)



f)



g)

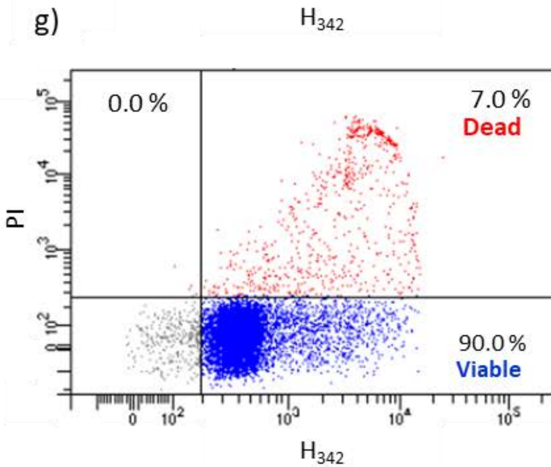


Figure 3.2. Gating strategy for assessment of the viability of subpopulations of *C. quadricarinatus* spermatozoa by Hoechst 33342/propidium iodide staining and flow cytometry. (a) FSC-area and FSC-height were used to exclude debris and aggregates; (b) the sperm population was gated using an FSC-A and SSC-A contour plot. An H_{342}/PI scatter plot was then used to identify the following: (c) unlabeled sperm cells, U_1, (d) H_{342} single-stained positive control (H_{342}^+), (e) fixed and permeabilised PI single-stained positive control (PI^+), (f) fixed and permeabilised double-stained H_{342}/PI-positive control (H_{342}^+/PI^+), and (g) a representative test sample showing subpopulations of viable and dead spermatozoa. FSC, forward scatter; H_{342}, Hoechst 33342; PI, propidium iodide; SSC, side scatter.

3.3.5. DNA fragmentation assessment

DNA fragmentation was assessed using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method, with minor modifications to the manufacturer's protocol (*In Situ* Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Mannheim, Germany), and the methods described by Peña et al. (2019) and Marc et al. (2021). For each male redclaw, the following control and test samples were prepared, with each containing 1.0×10^5 spermatozoa: unlabeled controls with and without H_{342} staining (U_1 & U_2, respectively), negative controls in label solution only (without terminal transferase) with and without H_{342} stain (N_1 & N_2, respectively), and *DNase*-treated FITC-positive controls with and without H_{342} stain (P_1 & P_2, respectively; Peña et al., 2019; Marc et al., 2021). Controls and test samples were fixed one hour after generating single-cell suspension and on the same day as sperm viability testing. Fixation was performed with constant agitation (450 rpm) using freshly prepared 4 % formaldehyde (dissolving paraformaldehyde in CFS solution, pH 7.4) for 1 h (RT). Samples were centrifuged (200 x *g*, 5 min), and sperm pellets were washed

twice with CFS solution. Sperm cells were permeabilised with 0.1 % Triton X-100 in 0.1 % sodium citrate (2 min, 4 °C), centrifuged (200 x *g*, 5 min), and then re-suspended in 200 µl CFS solution and stored at 4 °C overnight.

Fixed and permeabilised positive controls (P_1 & P_2) were established by incubating samples with micrococcal nuclease (10 U/µl of *DNase* 1 recombinant dissolved in Roche Buffer 2, RB_2, containing 10 mM Tris-HCl, 10 mM NaCl, 5 mM $MnCl_2 \cdot 4H_2O$, 0.1 mM $CaCl_2$, 25 mM KCl at a final concentration of 0.10 U/µl *DNAse*, pH 7.4) for 40 min to induce DNA strand breaks. Samples were washed twice with CFS solution (200 x g, 5 min) prior to TUNEL labelling.

For DNA labelling, unlabeled controls (U_1 & U_2) were re-suspended in 50 µl CFS solution, negative controls (N_1 & N_2) were re-suspended in 50 µl label solution (without terminal transferase), and positive controls (P_1 & P_2), as well as all test samples, were re-suspended in 50 µl TUNEL reaction mixture. All samples were then incubated for 90 min at RT, protected from light. U_2, N_2, P_2 and all test samples were counterstained with 5 µg/mL H_{342} (40 min, RT, protected from light). The *DNAse*-treated FITC and H_{342} positive control (P_2) induced 95.7 ± 1.9 % sperm DNA damage (mean ± SEM). Prior to flow cytometry, all samples were washed twice with CFS solution and re-suspended with 2 mM EDTA in CFS solution to a final volume of 1 mL. Using this method, the nucleus of spermatozoa with intact DNA stain blue (H_{342}^+/ $FITC^-$), while those with fragmented DNA stain blue-green (H_{342}^+/ $FITC^+$).

Staining specificity was confirmed by fluorescent microscopy (Olympus BX53/CoolLED pE-300 W fluorescent microscope, Tokyo, Japan) using the blue (Ex/Em = 345/478) and green (Ex/Em = 495/518) channels (Figures 3.1 c – d), prior to flow cytometry to assess the

percentage of DNA-damaged spermatozoa among the total number of identified spermatozoa within the gated area (Figure 3.3 i).

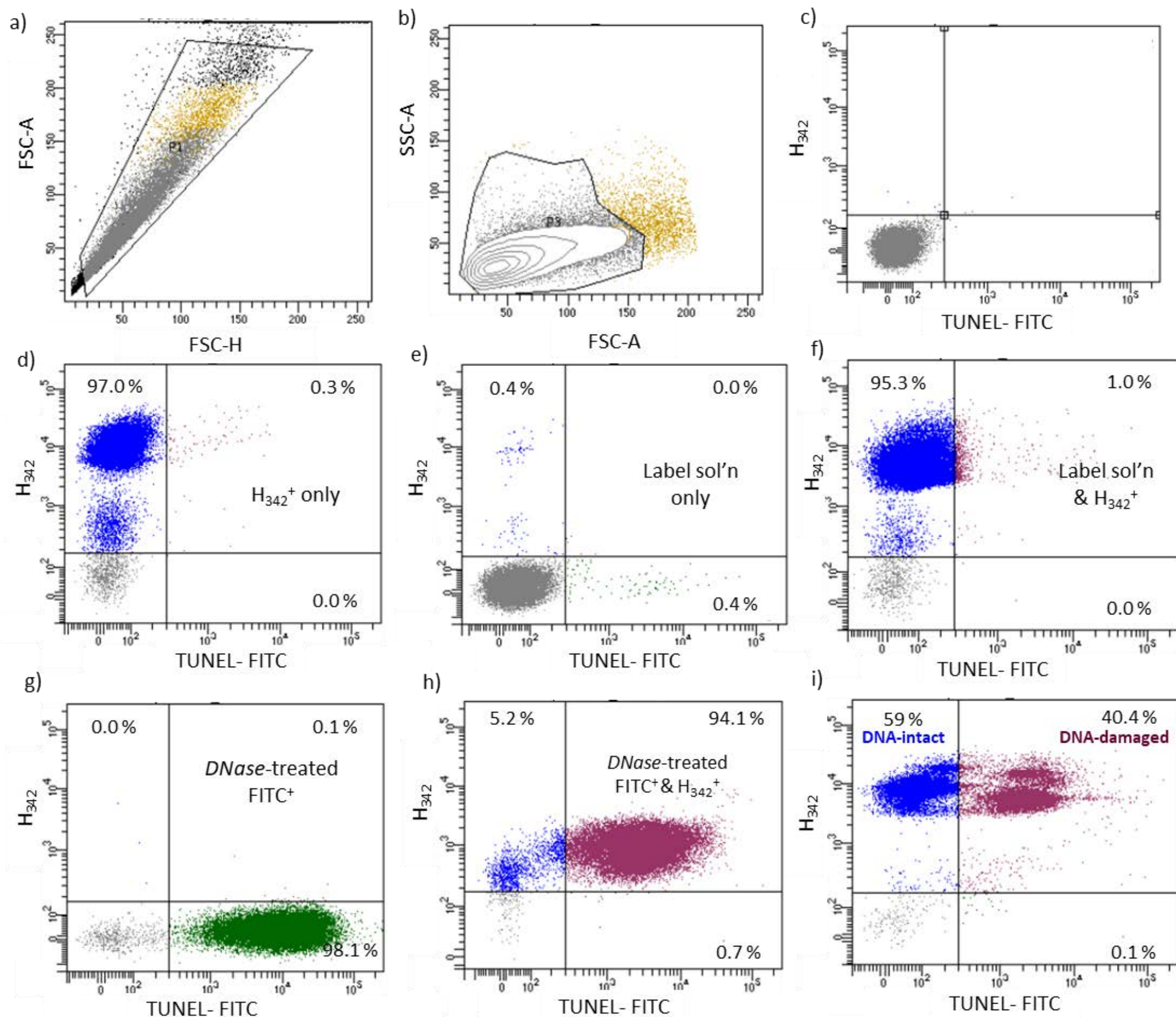


Figure 3.3. Gating strategy for assessment of sperm DNA fragmentation of subpopulations of *C. quadricarinatus* spermatozoa by TUNEL and flow cytometry. (a) FSC-Area and FSC-Height were used to exclude debris and aggregates; (b) the sperm population was gated using an FSC-A and SSC-A contour plot. An H_{342}/TUNEL-FITC scatter plot was then used to identify the following: (c) unlabeled sperm cells, U_1; (d) unlabeled control with H_{342}, U_2; (e) negative control in label solution, N_1; (f) negative control in label solution with H_{342}, N_2; (g) *DNase*-treated FITC positive control, P_1; (h) *DNase*-treated FITC positive control with H_{342}, P_2; and (i) a representative test sample showing subpopulations of intact and DNA-damaged

spermatozoa. FITC, fluorescein isothiocyanate; FSC, forward scatter; H_{342}, Hoechst 33342; SSC, side scatter.

3.3.6. Flow cytometry

To quantify viability and DNA fragmentation, 1.0 x 10^5 spermatozoa per sample were acquired on a FACS Canto™ II flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with blue (488 nm), red (633 nm) and violet (405 nm) lasers. Instrumental sensitivity was monitored using the BD Cytometer Setting & Tracking (CS&T) system and beads (Lot: 30381). A total of 10,000 events were collected per sample. Cytometric data analysis was performed using FACSDiva software version 6.1.3 (BD Biosciences).

Spermatozoa were distinguished from debris and aggregates using forward scatter-area (FSC-A) *vs* forward scatter-height (FSC-H) properties, followed by FSC-A and side-scatter-area (SSC-A) signals (Figures 3.2 a – b and Figures 3.3 a – b). Positive and negative controls were used to define gating regions and single colour controls for spectral overlap compensation for H_{342}/PI (sperm viability; Figures 3.2 c – g) and H_{342}/TUNEL-FITC (DNA fragmentation; Figures 3.3 c – i). Thresholds were set to 0.5 % using H_{342} positive control and 1.0 % using negative control in label solution with H_{342} (N_2) for sperm viability and DNA fragmentation assays, respectively. The photomultiplier tubes (PMT) detector of the flow cytometer was set to the following voltages: FSC = 324 V, SSC = 345 V, Hoechst filter = 250 V, FITC filter = 320 V and PI filter = 390 V.

3.3.7. Determination of total potential fertile sperm concentration (TPFSC)

The total potential fertile sperm concentration was calculated using the formula:

$$TPFSC = [SC - (SC * DF)] * VS$$

where,

SC = sperm concentration (cells/mL)

DF = DNA fragmented spermatozoa (%)

VS = viable spermatozoa (%)

3.3.8. Statistical analyses

Statistical analyses were performed using custom scripts in RStudio version 4.1.2 (R Core Team, 2022 ; RStudio Inc., Boston, MA, USA). The relationship between morphological features such as body and spermatophore weights and sperm quality (sperm concentration, count, viability, DNA fragmentation, and TPFSC) was evaluated to establish baseline data for male fertility. The relationship between variables was assessed by linear regression analysis and was considered significant at $p \leq 0.05$. The strength of significant relationships was summarised using Pearson's correlation coefficient (r) based on the BMJ criteria (Swinscow, 1997). Due to the significant correlation between body morphometrics (CL, CW, and TL) and body weight (r = 0.95, 0.98, 0.94, respectively), the relationship between male redclaw body weight and sperm quality was assessed. Diagnostic plots were employed to detect heterogeneity of variance and non-normal distribution of data residuals. Log transformation was used to correct non-normality or heteroscedasticity prior to the regression analysis of sperm concentration, count and TPFSC, while logit transformation was

used for sperm viability and DNA fragmentation. The raw data were used in plotting regression graphs between body and spermatophore weights and sperm quality indicators with significant relationships. Data variables were summarised as mean ± SEM.

3.4. Results

3.4.1. Reproductive data of male fertility for redclaw crayfish

After electroejaculation, extruded spermatophores were intact, with a sticky and sponge-like consistency. Within an hour of collection, the spermatophore solidified, forming an intense white structure that persisted for up to 24 h (Figure 3.4 a). Spermatozoa were embedded in the primary spermatophore layer, which could be differentiated from the translucent secondary spermatophore layer after 24 h and confirmed by H_{342}^{+} staining (Figure 3.4 b). Primary and secondary spermatophore layers and spermatozoa became distinctly visible under phase contrast without the aid of H_{342} staining after 96 h when suspended in CFS solution (Figures 3.4 c – d). Melanised spermatophores were not observed in the current study.

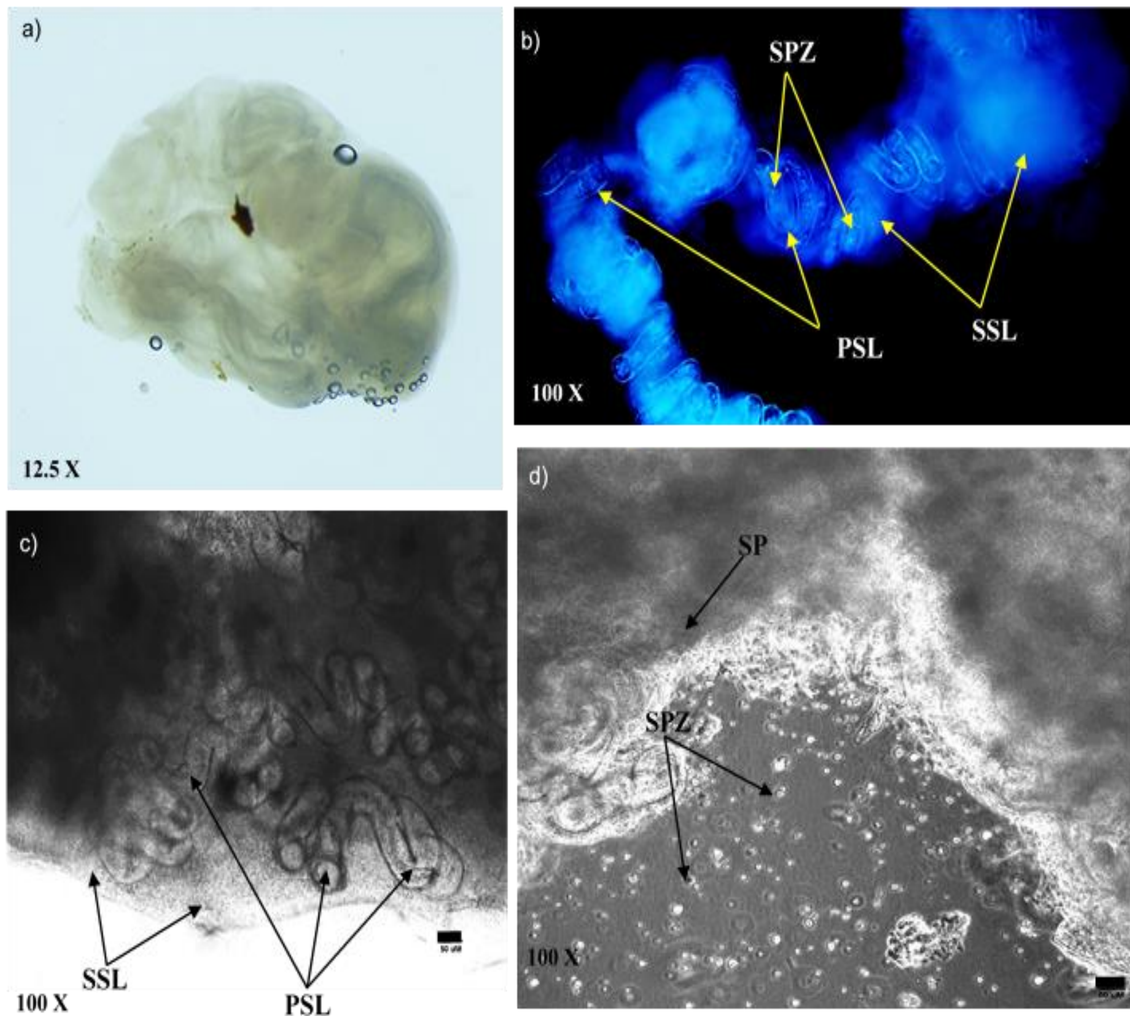


Figure 3.4. Sub-structure of the extruded spermatophore (SP) of *C. quadricarinatus*. (a) Intact spermatophore 10 min after extrusion (stereo microscopy); (b) H_{342}-stained spermatophore showing distinct primary and secondary layers 24 h after extrusion (inverted fluorescent microscopy); c) spermatophore showing distinct primary and secondary layers after 96 h (phase contrast); (d) spermatozoa liberated from a spermatophore after 96 h by gentle pipetting (phase-contrast). H_{342}, Hoechst 33342; PSL, primary spermatophore layer; SSL, secondary spermatophore layer; SP, spermatophore; SPZ, spermatozoa; scale bar = 50 µm (c and d).

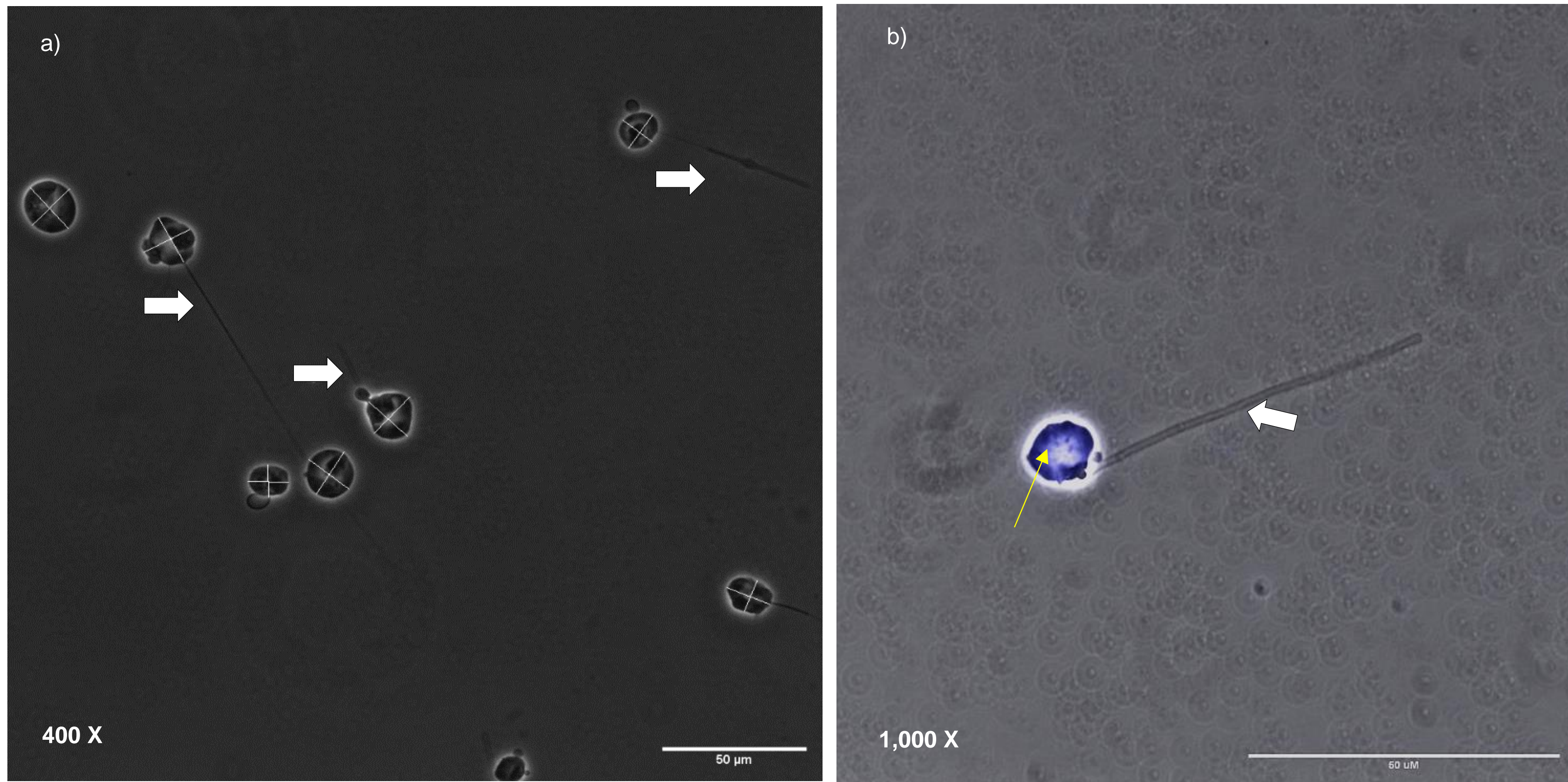


Figure 3.5. Phase contrast micrographs of *C. quadricarinatus* spermatozoa. (a) Measurement of the sperm head (length and width) and presence of a putative tail-like structure (white arrows) (b) Hoechst 33342 stained nuclear area (yellow arrow) of spermatozoa and putative tail-like structure (white arrow). Scale bar = 50 µm.

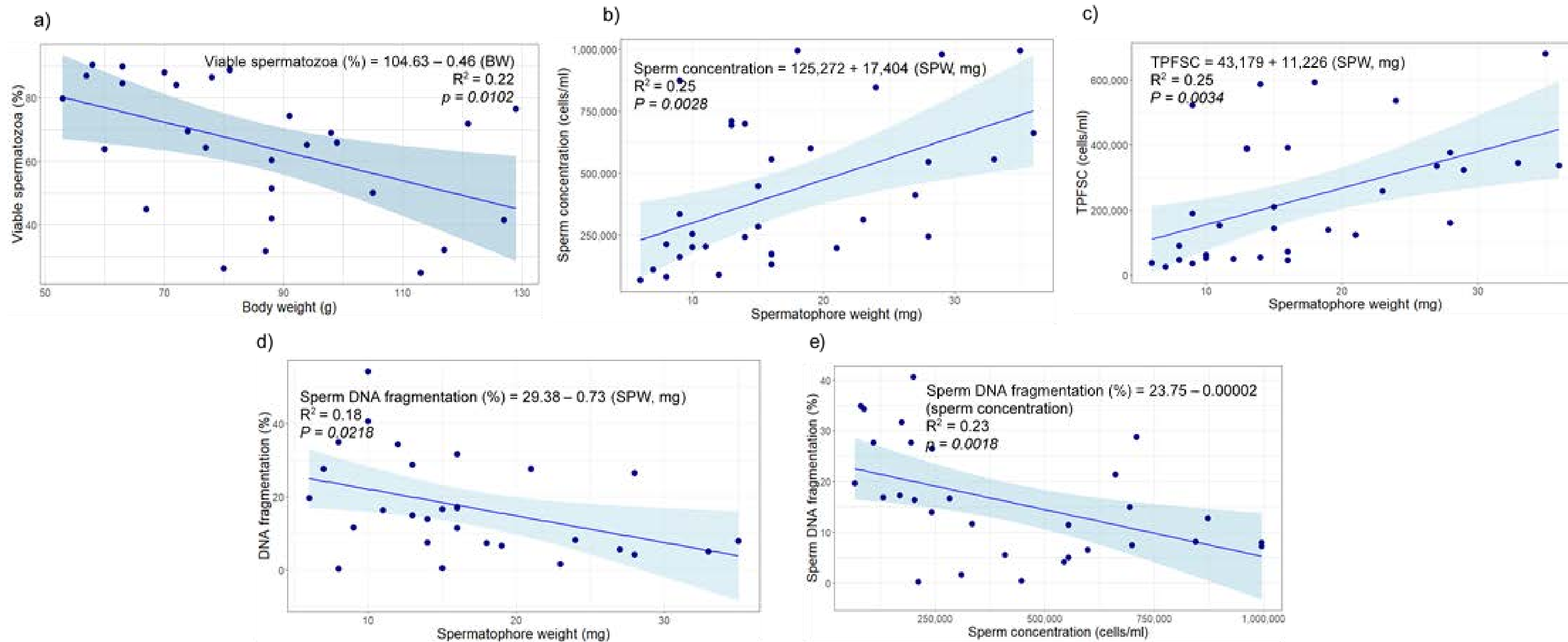


Figure 3.6. Linear regressions with a 95% confidence interval (blue band) between (a) body weight and sperm viability, (b) spermatophore weight and sperm concentration, (c) spermatophore weight and total potential fertile sperm concentration, (d) spermatophore weight and sperm DNA fragmentation, and (e) sperm concentration and DNA fragmentation. All regressions are significant at $p \leq 0.05$. BW, body weight; SPW, spermatophore weight; TPFSC, total potential fertile sperm concentration.

Redclaw spermatozoa were immotile, highly sticky, and appeared to have three distinct morphological features: (i) an elliptical sperm head that consists of a main body enclosing a decondensed nucleus in the posterior aspect of the cell (Figures 3.1 b & 3.5 b - yellow arrows), (ii) an acrosome-like cap (Figure 3.1 b - asterisk,), and the presence of a putative tail-like structure in many, but not all spermatozoa (white arrows in Figures 3.5 a – b). Based on examination of > 200 spermatozoa from 10 randomly selected males, 19.6 ± 1.2 % (mean ± SEM) of the redclaw sperm population were identified as having this presumptive tail-like structure. Spermatozoa with the tail-like structure had a head length of 13.7 ± 0.5 µm, a head width of 11.7 ± 0.5 µm, and a tail length of 47.4 ± 3.3 µm (mean ± SEM; n = 100 spermatozoa). Sperm parameters, including head length, width, area, ratio, perimeter, surface area, and volume, are shown in Table 3.1. Several parameters (head area, surface area and volume) were highly variable between individual spermatozoa.

Values of sperm quality for wild-caught adult redclaw are shown in Table 3.2. Similar to individual spermatozoa, a high degree of variability was also observed for many sperm quality parameters (sperm concentration, sperm count, DNA damage and TPFSC) between individual males. With the exception of sperm viability (which was moderately negatively correlated, $p = 0.010$), there was no significant relationship between body weight and any sperm quality parameters, including spermatophore weight (Table 3.3). Sperm viability decreased by 0.47 % for every gram increase in the body weight of male redclaw crayfish (Figure 3.6 a).

By contrast, spermatophore weight was moderately and positively correlated with sperm concentration ($p = 0.001$) and total potential fertile sperm concentration (TPFSC, $p < 0.001$) and moderately negatively associated with DNA fragmentation ($p = 0.022$) but not sperm

viability (Table 3.3). Sperm concentration and TPFSC increased by 17,404 and 11,226 cells/ml, respectively, for every milligram increase in spermatophore weight (Figure 3.6 b – c). In contrast, sperm DNA fragmentation decreased by 0.73 % for every milligram increase in spermatophore weight (Figure 3.6 d).

Sperm concentration was moderately and negatively correlated with DNA fragmentation ($p = 0.008$; Table 3.3). Sperm DNA fragmentation decreased by 0.00002 % on average in every unit increase of sperm concentration (cells/ml; Figure 3.6 e). No direct association was found between sperm viability and concentration, count, and DNA fragmentation in wild-caught male redclaw spermatozoa (Table 3.3).

Table 3.1. Characterisation of sperm morphology in wild-caught adult redclaw crayfish (*C. quadricarinatus*).

Sperm morphology	mean ± SEM (n = 300 spermatozoa)	Range (min-max)
Head length (μm)	16.3 ± 0.3	5.2 –36.1
Head width (μm)	14.2 ± 0.3	4.5 – 33.7
Head area (μm^2)	204.5 ± 8.8	19.2 – 948.6
Head ratio (length/width)	1.18 ± 0.01	0.9 – 2.1
Head perimeter (μm)	68.2 ± 1.4	22.2 – 154.6
Head surface area (μm^2)	1,786.3 ± 79.1	1.1 – 8,630.8
Head volume (μm^3)	2,495.9 ± 177.0	57.4 – 21,130.9

Table 3.2. Reproductive values of sperm quality for wild-caught adult redclaw crayfish (*C. quadricarinatus*).

Parameter	mean ± SEM (n = 33 animals)	Range (min-max)
Spermatophore weight (mg)	17.2 ± 1.5	6.0 – 36.0
Sperm concentration (x 10^4 cells/ml)	42.5 ± 5.1	6.6 – 99.5
Sperm count (x 10^6 cells/g SP)	26.1 ± 3.3	7.4 – 96.9
Viable spermatozoa (%)	65.2 ± 3.9	24.8 – 90.3
DNA-damaged spermatozoa (%)	17.2 ± 2.5	0.2 – 54.2
TPFSC (x 10^4 cells/ml)	23.6 ± 3.4	2.5 – 68.0

Table 3.3. Relationship between sperm quality parameters and body and spermatophore weights in wild-caught adult redclaw crayfish (*C. quadricarinatus*).

Explanatory variable	In relation to sperm quality parameter	R^2	*p-value*	Pearson's correlation coefficient (*r*)	Positive (+), negative (-) or no association?
Body weight (g)	Sperm concentration (cells/ml)	0.09	0.086		No
	Sperm count (cells/g)	0.05	0.193		No
	Sperm viability (%)	**0.22**	**0.010**	**- 0.47**	**Moderate (-)**
	Sperm DNA fragmentation (%)	0.03	0.356		No
	Total potential fertile sperm concentration (log-transformed data; TPFSC/ml)	0.005	0.691		No
	Spermatophore weight (g)	0.05	0.197		No
Spermatophore weight (g)	Sperm concentration (log-transformed data; cells/ml)	**0.30**	**0.001**	**0.55**	**Moderate (+)**
	Sperm viability (%)	0.06	0.188		No
	Sperm DNA fragmentation (%)	**0.18**	**0.022**	**- 0.42**	**Moderate (-)**
	Total potential fertile sperm concentration (log-transformed data; TPFSC/ml)	**0.33**	**< 0.001**	**0.58**	**Moderate (+)**

Explanatory variable	In relation to sperm quality parameter	R^2	*p-value*	Pearson's correlation coefficient (*r*)	Positive (+), negative (-) or no association?
Sperm concentration (cells/ml)	Sperm viability (%)	0.01	0.5131		No
	Sperm DNA fragmentation (%)	**0.23**	**0.008**	**- 0.48**	**Moderate (-)**
Sperm count (cells/g SP)	Sperm viability (logit-transformed data; %)	0.001	0.8719		No
	Sperm DNA fragmentation (%)	0.08	0.1267		No
Sperm viability (%)	Sperm DNA fragmentation (logit-transformed data; %)	0.03	0.336		No

3.5. Discussion

Using a combination of conventional and advanced functional sperm quality diagnostic tools, we describe, for the first time, reproductive data on sperm quality for wild-caught redclaw crayfish (*C. quadricarinatus*) from North Queensland, Australia. A novel putative tail-like structure, which has not previously been reported for redclaw spermatozoa, was identified. This study describes the electroejaculation of spermatophores from redclaw crayfish and sperm extraction without using enzymatic/chemical treatment or mechanical shearing. There was a high rate of sperm DNA fragmentation, low sperm viability, and a broad variability of sperm quality among males. Additionally, lower sperm viability was observed in heavier animals. This study found that heavier spermatophores had higher sperm concentration, less DNA damage, and improved total potential fertile sperm concentration (TPFSC). TPFSC

refers to the concentration of viable spermatozoa with intact DNA. The term "potential fertile" was applied in this study since other predictive biomarkers, including sperm mitochondrial function and acrosome capacitation, could also contribute to spermatozoa's fertilising capacity but require optimisation in redclaw spermatozoa. In the future, assessment of more variables that affect sperm quality may provide a more accurate assessment of TPFSC.

Electroejaculation, followed by spermatophore suspension in CFS solution, was a reliable and non-lethal method to extrude and process spermatophores from wild-caught male redclaw. The characteristics of the spermatophore after extrusion were similar to those described by López Greco and Lo Nostro (2008), where the spermatophore initially solidified, then disintegrated over time, liberating spermatozoa after 96 h. In the wild, male crayfish deposit the spermatophore on the female's abdomen near her fifth pereiopods during copulation; when the spermatophore breaks down, the female redclaw manipulates the spermatophore to release spermatozoa to fertilise eggs (Jones et al., 1996; López Greco and Lo Nostro, 2008). In this study, the H_{342} staining of the spermatophore 24 h post extrusion confirmed that the first and second layers of the spermatophore are permeable, and this characteristic likely contributed to our ability to prepare a single-sperm cell suspension after manually cutting the spermatophore into multiple sections and releasing spermatozoa by gentle pipetting.

In crustaceans, single sperm suspensions have been achieved using mechanical disruption of spermatophore after collection, involving maceration and repeat pipetting in a semen extender solution to disrupt spermatophore walls, or by gentle homogenisation using a tissue grinder (Leung-Trujillo and Lawrence, 1987; Bray and Lawrence, 1998; Bart et al., 2006;

Bugnot and López Greco, 2009; Gwo, 2009; Feng et al., 2019). In addition, many protocols incorporate enzymatic and chemical digestion using pronase (Bhavanishankar and Subramoniam, 1997), trypsin (Chao et al., 2009; Wang et al., 2015), and sodium hydroxide (Sato et al., 2008) while suspending spermatophores at 4 ºC and in sperm extender for a period of time, followed by mechanical homogenisation. However, mechanical, enzymatic, or chemical digestion of spermatophores to produce a single-sperm suspension can induce the formation of spermatozoa aggregates and adhesive matrices potentially impacting sperm quality and its usefulness in downstream cryopreservation and artificial insemination procedures (Bhavanishankar and Subramoniam, 1997; Sato et al., 2004; Sato et al., 2005; Sato et al., 2008; Erraud et al., 2018). Here, we describe a gentle extraction method that mimics the natural process of progressive hydration of the spermatophore matrix to liberate individual redclaw spermatozoa, without mechanical shearing or enzymatic and chemical digestion.

The sperm count (~ 3.0×10^7 cells/g spermatophore) yielded from this study is within the range of sperm counts reported by Bugnot and López Greco (2009; 10^7 – 10^9 spermatozoa/ one cm DVD section) in redclaw crayfish, though the two studies have different methods of estimating sperm concentration. In this study, the estimation of sperm count was based on the electroejaculated spermatophore and is expressed per gram of spermatophore. In contrast, Bugnot and López Greco (2009) removed spermatophore via terminal dissection and estimated sperm count per one-centimetre distal vas deferens (DVD) section under light microscopy. Further optimisation is needed for the handling and dissolution of spermatophores, including appropriate holding media, in order to improve sperm yields in redclaw crayfish and examine the effect of the extraction method on sperm quality.

In decapod crustaceans, abnormal sperm morphology is used to indicate potential subfertility, or an atypical outcome of spermatogenesis (Leung-Trujillo and Lawrence, 1987; Meunpol et al., 2005; Leelatanawit et al., 2014; Harlıoğlu et al., 2018; Pérez-Rodríguez et al., 2019; Peña-Almaraz et al., 2022). To our knowledge, this is the first comprehensive description of sperm morphology and quality in wild-caught, freshwater redclaw crayfish. The range of redclaw crayfish spermatophore weights (6 – 36 mg) collected via electroejaculation in the current study is comparable with those of wild-caught marine shrimps (10 – 93 mg; Rendón Rodríguez et al., 2007; Vuthiphandchai et al., 2007). Redclaw spermatozoa (head length 16.3 ± 0.3 µm x width 14.2 ± 0.3 µm) appear to be smaller than other decapods studied to date, including wild-caught marine shrimp, *P. japonica* (52 – 55 µm head length x 24 – 26 µm width; Kim et al., 2003). To date, sperm morphology alone has proven inaccurate for distinguishing normal from abnormal redclaw spermatozoa. Previous studies report that redclaw spermatozoa are irregular in shape, lack spikes or true flagella, and are immotile (Beach and Talbot, 1987; An et al., 2011; Kouba et al., 2015). In contrast, we identified three distinct morphological features of redclaw spermatozoa: different sizes of an elliptical sperm head that consists of a main body enclosing a decondensed nucleus, an acrosome-like cap, and the occasional presence of a putative tail-like structure (Figure 3.5).

Our observation of the presumptive tail-like structure in redclaw spermatozoa may be due to more gentle methods of spermatophore processing and visualisation of spermatozoa that may help protect these delicate structures. Previous freshwater crayfish studies have evaluated morphology in fixed and dehydrated spermatozoa using histology and transmission electron microscopy (TEM; Beach and Talbot, 1987; An et al., 2011; Kouba et al., 2015) or scanning electron microscopy (SEM; Niksirat et al., 2014), rather than phase-contrast microscopy. These processing methods can potentially alter cell size (shrinkage) and distort sperm shape,

which could detach or reduce the visibility of tail-like structures (Cabrita et al., 2009). Further studies are needed to confirm if the tail-like structure is composed of microtubules, or is in fact a type of radial arm involved in the acrosome reaction associated with fertilisation (Aquino et al., 2022), as not all spermatozoa appear to possess the tail-like structure (Figure 3.5 a). Previous studies in redclaw crayfish and other *Cherax* species have not reported the presence of a true flagellum or radial arms (Beach and Talbot, 1987; An et al., 2011; Kouba et al., 2015). However, the presence of acrosomal horns or spikes that are thought to have a final role in sperm maturation and acrosome reaction has been proposed in other genera of freshwater crayfish (Niksirat et al., 2013; Niksirat et al., 2014).

To our knowledge, this is the first report on the application of flow cytometry to evaluate sperm viability and DNA fragmentation in freshwater crayfish. Assessment of plasma membrane integrity is used as a biomarker for sperm viability, where any damaged sperm cell membrane is equivalent to non-functional and/or dead spermatozoa (Cabrita et al., 2009). Lezcano et al. (2004) reported that identifying cell viability in spermatozoa from marine shrimp using nuclear staining coupled with flow cytometry, is more sensitive and less prone to inter-observer errors than optical microscopy. In the current study, the mean sperm viability was 65.2 ± 3.9 %; however, this varied widely between animals (24.8 – 90.3 %). Our viability findings were lower than the values of 97 to 99 % reported in redclaw crayfish by Bugnot and López Greco (2009), and in other freshwater crayfish (78 – 83 %, *Pontastacus leptodactylus*; Farhadi et al., 2019), where sperm viability was estimated by methylene blue/eosin-nigrosin staining under light microscopy. However, our findings are comparable with those of Galeotti et al. (2012), who reported an average of 68.0 ± 2.4 % viable spermatozoa, ranging from 13.2 – 91.6 %, in white-clawed crayfish (*Austropotamobius italicus*), and where sperm viability was evaluated using dual nuclear staining under

fluorescent microscopy. In this study, we used similar techniques to Galeotti et al. (2012), except that spermatozoa viability was assessed using dual fluorochrome nuclear staining followed by flow cytometry, which provides a highly specific method for determining the status of plasma membrane integrity. This method also allows the assessment of a higher number of spermatozoa (> 10,000 cells) per sample in a short time (~ 1 min) compared to 100 – 200 cells when using microscopy (Garner et al., 1986; Liu et al., 2007; Cabrita et al., 2009). In other decapod crustaceans collected from the wild, sperm viability has been documented to be 63 to 65 % for freshwater prawns (*Macrobrachium americanum*; Pérez-Rodríguez et al., 2019) and 53 to 99 % for penaeid shrimps (Perez-Velazquez et al., 2001; Rendón Rodríguez et al., 2007; Silva et al., 2015).

Analysis of sperm DNA fragmentation is an emerging diagnostic tool to estimate the fertility potential of male broodstock in crustacean aquaculture, with only some studies documented in crabs (Ma et al., 2013; Noor-Hidayati et al., 2014; Li et al., 2016) and shrimps (Erraud et al., 2018; Feng et al., 2018; Erraud et al., 2019; Feng et al., 2019; Duangjai et al., 2023). Although spermatozoa may appear morphologically normal and retain the capacity to fertilise eggs, they can contain fragmented DNA, which has the potential to arrest or impair embryo and larval development and lower yield (Bakos et al., 2008; Castelo-Branco et al., 2018; Peña et al., 2019). Duangjai et al. (2023) reported that conventional sperm quality parameters failed to reflect differences in the fertility of male decapod crustaceans and suggested that measuring sperm DNA damage could serve as a more sensitive diagnostic tool. Several DNA fragmentation assays have been applied to crustaceans (Noor-Hidayati et al., 2014; Shu and Zhang, 2017; Erraud et al., 2018; Feng et al., 2018; Erraud et al., 2019; Feng et al., 2019) and finfishes (Pérez-Cerezales et al., 2010; Cabrita et al., 2014; Marc et al., 2021). In the current study, we observed 17.2 ± 2.5 % DNA-damaged spermatozoa in wild-caught redclaw

crayfish using TUNEL staining, coupled with flow cytometry. Despite different methods, our findings are comparable to the average sperm DNA damage reported in whiteleg shrimp (*Litopenaeus* vannamei) evaluated using a Comet assay (20.7 ± 3.2 %) and freshwater crabs (*Sinopotamon henanense*) evaluated by DNA-protein crosslink quantification (21 %; Li et al., 2016). The sperm DNA fragmentation found in redclaw, and other decapod crustaceans is much higher than observed in other vertebrate species (Pérez-Cerezales et al., 2009; Van den Berghe et al., 2018; Peña et al., 2019), which could partly explain the low survival of juveniles yielded by the traditional hatchery-nursery system in earthen ponds. Nevertheless, viability and DNA fragmentation in redclaw spermatozoa are highly variable, 24.8 – 90.3 % and 0.2 – 54.2 %, respectively, suggesting that it may be feasible to apply a combination of conventional and advanced tools to rank and select highly fertile males as broodstock, that is, by retaining only males with > 80 % sperm viability and < 3 % DNA fragmentation to ensure successful breeding and production in commercial redclaw hatchery. Validation of this concept will, however, require the conduct of studies investigating the effects of these variables on fertility.

In this study, sperm viability was negatively and significantly associated with the body weight of wild-caught male redclaw (Table 3.3; Figure 3.6 a) but not with spermatophore weight. Furthermore, while DNA fragmentation had no significant association with body weight ($p > 0.05$), it was inversely proportional to spermatophore weight ($p < 0.05$; Table 3.3; Figure 3.6 d). The negative association found between sperm DNA fragmentation and spermatophore weight could be attributed to the protective function of heavier spermatophores to better shield spermatozoa from unfavourable conditions, or enhanced spermatogenic function or nutritive status, which perhaps resulted in spermatophores of greater weight with better sperm quality, or possibly confer other unknown advantages to

sperm quality. Future studies using larger sample sizes of wild-caught and farmed males must validate our findings.

In terrestrial production animals, there is a direct relationship between animal body weight and sperm number (since large males typically have larger testes; Rijsselaere et al., 2007; Turri et al., 2016; Wang et al., 2017; Tesi et al., 2018; Kozopas et al., 2020). In the present study, male body weight was a poor predictor of sperm quality across almost all parameters ($p > 0.05$; Table 3.3). This contrasts Bugnot and López Greco (2009) findings, which reported a significant and positive association between body weight and sperm count in the same species. These conflicting findings may be attributed to differences in study design in terms of animal source, range of body weights investigated, and method of spermatophore collection. Bugnot and López Greco (2009) used captive-reared broodstock with a wider range of body weights (8.6 – 270.6 g), with spermatophore removed from the distal vas deferens (DVD) by post-mortem dissection and sperm count calculated per one centimetre DVD. Studies in several aquaculture species demonstrate a gradual decline in sperm counts in broodstock compared to wild-caught individuals (Leung-Trujillo and Lawrence, 1987; Rendón Rodríguez et al., 2007; Silva et al., 2015; Gilroy and Litvak, 2019; Sheikh et al., 2019). The difference in sperm quality between captive-reared and wild-caught breeders may be attributed to the animals' environmental conditions, nutrition, and stress-mediated changes in spermatogenesis, with further research in this field warranted.

In some species, ejaculate volume is directly related to the concentration, number and even motility of spermatozoa (since larger ejaculates typically contain more sperm and accessory gland secretions to power their motility; Gilroy and Litvak, 2019; Górski et al., 2021). In the present study, spermatophore weight was a reasonable predictor of sperm quality parameters,

including sperm concentration, TPFSC and sperm DNA fragmentation (Table 3.3). Thus, for wild-caught male redclaw, heavier spermatophores were associated with more concentrated sperm and TPFSC and slightly lower DNA damage (Figures 3.6 b – d). Correlation analysis revealed a negative and moderate association between sperm concentration and DNA fragmentation (Table 3.3; Figure 3.6 e), which is unequivocally parallel with recent reports (Yang et al., 2019; Liu et al., 2023). Studies in other decapod crustaceans have reported a relationship between spermatophore size/weight and melanisation, sperm quality, fertilisation, and fecundity (Leung-Trujillo and Lawrence, 1987; Sato et al., 2008; Braga et al., 2010; Braga et al., 2018; Pérez-Rodríguez et al., 2019; Jiang et al., 2020). According to a study by Sato et al. (2008), a larger spermatophore volume would contain more spermatozoa. Pérez-Rodríguez et al. (2019) reported that spermatophore weight determines sperm counts in river prawns (*Macrobrachium americanum*), emphasising that small-sized spermatophores have a lower number of spermatozoa for oocyte fertilisation. In spiny lobsters (*Jasus* and *Panulirus* species), more eggs were fertilised in a breeding trial with larger spermatophore size (MacDiarmid and Butler, 1999). In addition, spermatophore quality, such as melanised spermatophore, yields low sperm count and an increase in abnormal spermatozoa in marine shrimps (Braga et al., 2018). Deterioration of spermatophore quality, such as melanisation and decreasing weight over time, causes a decline in sperm quality and an indication of reproductive exhaustion (Leung-Trujillo and Lawrence, 1987; Braga et al., 2010; Jiang et al., 2020). Evaluating the weight and degree of melanisation of the redclaw spermatophore may offer an initial screening tool to predict sperm quality prior to more comprehensive diagnostic tests.

3.6. Conclusions

This study describes a method for preparing a single-sperm cell suspension of redclaw spermatozoa without enzymatic/chemical treatment or mechanical shearing. We report using conventional and advanced diagnostic tools to evaluate sperm quality in redclaw crayfish. We provide the first quantitative reproductive data on sperm function and morphometry, including identifying a putative tail-like structure in some spermatozoa. This study demonstrated that spermatophore weight might be a useful predictor of sperm quality to inform the selection of fertile broodstock males for use in intensive breeding and juvenile production. Lastly, this study reported enhanced strategies in harvesting spermatozoa that could be used to cryopreserve spermatozoa, and downstream use in artificial fertilisation for commercial redclaw aquaculture.

3.7. Acknowledgements

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3.8. References

An, C.-G., Weng, X.-L., Xu, Y.-Z., Fan, Y.-J., Zhao, Y.-L., 2011. Histological and ultrastructural studies on the male reproductive system and spermatogenesis in the red claw crayfish, *Cherax quadricarinatus*. J. Crust. Biol. 31(2), 223-230. https://doi.org/https://doi.org/10.1651/10-3342.1.

Aquino, J.I.L., Elliott, L., Zeng, C., Paris, D.B.B.P., 2022. Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation, and their potential application to decapod crustacean aquaculture. Rev. Aquac. 14(2), 848-889. https://doi.org/https://doi.org/10.1111/raq.12627.

Bakos, H.W., Thompson, J.G., Feil, D., Lane, M., 2008. Sperm DNA damage is associated with assisted reproductive technology pregnancy. Int. J. Androl. 31(5), 518-526. https://doi.org/10.1111/j.1365-2605.2007.00803.x.

Bart, A.N., Choosuk, S., Thakur, D.P., 2006. Spermatophore cryopreservation and artificial insemination of black tiger shrimp, *Penaeus monodon* (Fabricius). Aquac. Res. 37(5), 523-528. https://doi.org/https://doi.org/10.1111/j.1365-2109.2006.01460.x.

Beach, D., Talbot, P., 1987. Ultrastructural comparison of sperm from the crayfishes *Cherax tenuimanus* and *Cherax albidus*. J. Crust. Biol. 7(2), 205-218. https://doi.org/https://doi.org/10.2307/1548602.

Bhavanishankar, S., Subramoniam, T., 1997. Cryopreservation of spermatozoa of the edible mud crab *Scylla serrata* (Forskal). J. Exp. Zool. 277(4), 326-336. https://doi.org/https://doi.org/10.1002/(SICI)1097-010X(19970301)277:4%3C326::AID-JEZ6%3E3.0.CO;2-R.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165(3), 535-548. https://doi.org/https://doi.org/10.1016/j.ygcen.2009.02.011.

Braga, A., Lopes, D., Magalhães, V., Klosterhoff, M.C., Romano, L.A., Poersch, L.H., Wasielesky, W., 2018. Hemocytic melanization in shrimp spermatophores. Aquaculture 486, : 64-67. https://doi.org/https://doi.org/10.1016/j.aquaculture.2017.12.018.

Braga, A.L., Nakayama, C.L., Martins, J.G., Colares, E.P., Wasielesky, W., 2010. Spermatophore quality of the pink shrimp *Farfantepenaeus paulensis* (Decapoda, Dendrobranchiata) broodstock fed with different maturation diets. Aquaculture 307(1), 44-48. https://doi.org/https://doi.org/10.1016/j.aquaculture.2010.07.010.

Bray, W.A., Lawrence, A.L., 1998. Male viability determinations in *Penaeus vannamei*: evaluation of short-term storage of spermatophores up to 36 h and comparison of Ca-

free saline and seawater as sperm homogenate media. Aquaculture 160(1), 63-67. https://doi.org/https://doi.org/10.1016/S0044-8486(97)00225-1.

Bugnot, A.B., López Greco, L.S., 2009. Sperm production in the red claw crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). Aquaculture 295(3), 292-299. https://doi.org/https://doi.org/10.1016/j.aquaculture.2009.07.021.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V., 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432, : 389-401. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.04.034.

Cabrita, E., Robles, V., Herráez, M.P., 2009. Sperm quality assessment, in: Cabrita, E., Robles, V., Herráez, M.P. (Eds.), Methods in Reproductive Aquaculture: Marine and Freshwater Species. Biology Series. CRC Press (Taylor and Francis Group), Boca Raton, Florida, USA, pp. 93-148.

Castelo-Branco, T., Guerra, M.M.P., Soares, R., Peixoto, S., 2018. Sperm vitrification of *Litopenaeus vannamei*: effect of cryoprotectant solutions on sperm viability and spawning quality after artificial insemination. Aquac. Int. 26(3), 913-920. https://doi.org/https://doi.org/10.1007/s10499-018-0258-y.

Chao, N.-H., Chen, Y.-N., Shieh, W.Y., Lin, K.-J., 2009. Cryopreservation of spermatophores in the white shrimp (*Litopenaeus vannamei*). J. Taiwan Fish. Res. 17(2), 67-76.

De Baulny, B.O., Le Vern, Y., Kerboeuf, D., Maisse, G., 1997. Flow cytometric evaluation of mitochondrial activity and membrane integrity in fresh and cryopreserved rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Cryobiology 34(2), 141-149. https://doi.org/https://doi.org/10.1006/cryo.1996.1992.

Duangjai, E., Umyoo, P., Meunpol, O., Puanglarp, N., 2023. Sperm quality assessment of white shrimp (*Litopenaeus vannamei*) broodstock using comet assay. Trends Sci. 20(6), 4768. https://doi.org/10.48048/tis.2023.4768.

Erraud, A., Bonnard, M., Duflot, A., Geffard, A., Danger, J.-M., Forget-Leray, J., Xuereb, B., 2018. Assessment of sperm quality in palaemonid prawns using Comet assay: methodological optimization. Environ. Sci. Pollut. Res. 25(12), 11226-11237. https://doi.org/https://doi.org/10.1007/s11356-017-8754-6.

Erraud, A., Bonnard, M., Geffard, O., Coulaud, R., Poret, A., Duflot, A., Forget-Leray, J., Geffard, A., Xuereb, B., 2019. Signification of DNA integrity in sperm of *Palaemon serratus* (Pennant 1777): Kinetic responses and reproduction impairment. Mar. Environ. Res. 144, 130-140. https://doi.org/https://doi.org/10.1016/j.marenvres.2019.01.005.

FAO-FIGIS, 2019. Global fisheries and aquaculture production 1950 - 2017, Fisheries Global Information System (FIGIS). FI Institutional Websites. Statistics and Information Branch. Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153, Rome.

Farhadi, A., Harlıoğlu, M.M., Gür, S., 2019. Artificial extrusion of spermatophores for insemination of the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Aquac. Rep. 14(100200), 1-5. https://doi.org/https://doi.org/10.1016/j.aqrep.2019.100200.

Favret, K.P., Lynn, J.W., 2010. Flow-cytometric analyses of viability biomarkers in pesticide-exposed sperm of three aquatic invertebrates. Arch. Environ. Contam. Toxicol. 58(4), 973-984. https://doi.org/https://doi.org/10.1007/s00244-009-9410-z.

Feng, T., 2018. Spermatogenesis and Sperm Assessment in the Black Tiger Prawn, *Penaeus monodon*, School of Agriculture and Food Sciences. University of Queensland, Queensland, Australia, p. 195.

Feng, T., Gosálvez, J., Lopez-Fernandez, C., Arroyo, F., Paterson, B., Johnston, S., 2018. Sperm chromatin dispersion test (SCDt) for the assessment of sperm DNA fragmentation in black tiger prawn, *Penaeus monodon*. Aquaculture 491, : 281-288. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.03.041.

Feng, T., Paterson, B., Coman, G., Johnston, S., 2019. Evaluating the impact of moulting and chilled storage of spermatophores on the integrity of plasma membrane, acrosome and DNA of black tiger prawn (*Penaeus monodon*) spermatozoa. Aquac. Res. 50(1), 226-235. https://doi.org/https://doi.org/10.1111/are.13888.

Galeotti, P., Bernini, G., Locatello, L., Sacchi, R., Fasola, M., Rubolini, D., 2012. Sperm traits negatively covary with size and asymmetry of a secondary sexual trait in a freshwater crayfish. *PLoS One* 7(8), e43771-e43771. https://doi.org/https://doi.org/10.1371/journal.pone.0043771.

Garner, D.L., Pinkel, D., Johnson, L.A., Pace, M.M., 1986. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol. Reprod. 34(1), 127-138. https://doi.org/https://doi.org/10.1095/biolreprod34.1.127.

Gillan, L., Evans, G., Maxwell, W.M.C., 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63(2), 445-457. https://doi.org/https://doi.org/10.1016/j.theriogenology.2004.09.024.

Gilroy, C.E., Litvak, M.K., 2019. Swimming kinematics and temperature effects on spermatozoa from wild and captive shortnose sturgeon (*Acipenser brevirostrum*). Anim. Reprod. Sci. 204, 171-182. https://doi.org/https://doi.org/10.1016/j.anireprosci.2019.03.022.

Górski, K., Kondracki, S., Iwanina, M., Kordan, W., Fraser, L., 2021. Effects of breed and ejaculate volume on sperm morphology and semen parameters of boars. Anim. Sci. J. 92(1), e13629. https://doi.org/10.1111/asj.13629.

Graham, E.F., Schmehl, M.K.L., Nelson, D.S., 1980. Problems with laboratory assays, in: Proceedings Eighth NMB Tech. Conf. Al. Reprod. pp. 59-66.

Graham, J.K., Kunze, E., Hammerstedt, R.H., 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol. Reprod. 43(1), 55-64. https://doi.org/https://doi.org/10.1095/biolreprod43.1.55.

Gwo, J.-C., 2009. Methods for sperm collection, in: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in Reproductive Aquaculture. Taylor & Francis Group, United States of America, pp. 81-86.

Harlıoğlu, M.M., Farhadi, A., 2017. Factors affecting the reproductive efficiency in crayfish: implications for aquaculture. Aquac. Res. 48(5), 1983-1997. https://doi.org/https://doi.org/10.1111/are.13263.

Harlıoğlu, M.M., Farhadi, A., Gür, S., 2018. Determination of sperm quality in decapod crustaceans. Aquaculture 490, : 185-193. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.02.031.

Haubrock, P.J., Oficialdegui, F.J., Zeng, Y., Patoka, J., Yeo, D.C.J., Kouba, A., 2021. The redclaw crayfish: A prominent aquaculture species with invasive potential in tropical and subtropical biodiversity hotspots. Rev. Aquac. 13(3), 1488-1530. https://doi.org/https://doi.org/10.1111/raq.12531.

Hossain, M.S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A.P., Rodriguez-Martinez, H., 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian J. Androl. 13(3), 406-419. https://doi.org/https://doi.org/10.1038/aja.2011.15.

Irvin, S., Coman, G., Musson, D., Doshi, A., Stokes, C., 2018. Aquaculture viability. A technical report to the Australian Government from the CSIRO Northern Australia Water Resource Assessment, part of the National Water Infrastructure Development Fund: Water Resource Assessments, CSIRO, Australia.

Janny, L., Menezo, Y.J.R., 1994. Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. Mol. Reprod. Dev. 38(1), 36-42. https://doi.org/https://doi.org/10.1002/mrd.1080380107.

Jerry, D.R., 2001. Electrical stimulation of spermatophore extrusion in the freshwater yabby (*Cherax destructor*). Aquaculture 200(3), 317-322. https://doi.org/https://doi.org/10.1016/S0044-8486(01)00511-7.

Jiang, S., Liu, D., Zhou, F., Mo, X., Yang, Q., Huang, J., Yang, L., Jiang, S., 2020. Effect of vitamin E on spermatophore regeneration and quality of pond-reared, black tiger shrimp (*Penaeus monodon*). Aquac. Res. 51(6), 2197-2204. https://doi.org/https://doi.org/10.1111/are.14524.

Jones, C., 1990. The biology and aquaculture potential of the tropical freshwater crayfish, *Cherax quadricarinatus*. Department of Primary Industries Queensland, Brisbane, QLD, Australia.

Jones, C., 2000. Recommended practices for redclaw crayfish aquaculture based on research and development activities, 1988 through 2000. Northern Fisheries Centre, Department of Primary Industries and Fisheries, Cairns QLD 4870, Australia.

Jones, C., 2011. Cultured aquatic species information programme- *Cherax quadricarinatus*, Cultured Aquatic Species Information Programme, 13 January 2011 ed. FAO Fisheries and Aquaculture Department, Rome.

Jones, C.M., 1995. Production of juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae) I. Development of hatchery and nursery procedures. Aquaculture 138(1), 221-238. https://doi.org/https://doi.org/10.1016/0044-8486(95)00068-2.

Jones, C.M., Ruscoe, I.M., Queensland, F.F.a.A.C., 1996. Production technology for redclaw crayfish (*Cherax quadricarinatus*) : Final report. Freshwater Fisheries and Aquaculture Centre, Department of Primary Industries, Walkamin, QLD.

Jones, C.M., Valverde, C., 2020. Development of mass production hatchery technology for the redclaw crayfish, *Cherax quadricarinatus*. Freshw. Crayfish 25(1), 1 - 6. https://doi.org/10.5869/fc.2020.v25-1.001.

Kim, D.H., Jo, Q., Choi, J.H., Yun, S.J., Oh, T.Y., Kim, B.K., Han, C.-H., 2003. Sperm Structure of the Pandalid Shrimp *Pandalopsis Japonica* (Decapoda, Pandalidae). J. Crustac. Biol. 23(1), 23-32. https://doi.org/10.1163/20021975-99990311.

Kooda-Cisco, M.J., Talbot, P., 1983. A technique for electrically stimulating extrusion of spermatophores from the lobster, *Homarus americanus*. Aquaculture 30(1), 221-227. https://doi.org/https://doi.org/10.1016/0044-8486(83)90164-3.

Kouba, A., Niksirat, H., Bláha, M., 2015. Comparative ultrastructure of spermatozoa of the redclaw *Cherax quadricarinatus* and the yabby *Cherax destructor* (Decapoda, Parastacidae). Micron 69, : 56-61. https://doi.org/https://doi.org/10.1016/j.micron.2014.11.002.

Kozopas, N.M., Chornenka, O.I., Vorobets, M.Z., Lapovets, L.Y., Maksymyuk, H.V., 2020. Body Mass Index and Sperm Quality: Is there a Relationship? J. Hum. Reprod. Sci. 13(2), 110-113. https://doi.org/10.4103/jhrs.JHRS_15_20.

Kumar, M., Kumar, K., Jain, S., Hassan, T., Dada, R., 2013. Novel insights into the genetic and epigenetic paternal contribution to the human embryo. Clinics 68, 5-14. https://doi.org/https://doi.org/10.6061/clinics/2013(Sup01)02.

Leelatanawit, R., Uawisetwathana, U., Khudet, J., Klanchui, A., Phomklad, S., Wongtripop, S., Angthoung, P., Jiravanichpaisal, P., Karoonuthaisiri, N., 2014. Effects of polychaetes (*Perinereis nuntia*) on sperm performance of the domesticated black tiger shrimp (*Penaeus monodon*). Aquaculture 433, : 266-275. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.06.034.

Leung-Trujillo, J.R., Lawrence, A.L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65(3), 363-370. https://doi.org/https://doi.org/10.1016/0044-8486(87)90249-3.

Lewis, C., Ford, A.T., 2012. Infertility in male aquatic invertebrates: A review. Aquat. Toxicol. 120-121, : 79-89. https://doi.org/https://doi.org/10.1016/j.aquatox.2012.05.002.

Lezcano, M., Granja, C., Salazar, M., 2004. The use of flow cytometry in the evaluation of cell viability of cryopreserved sperm of the marine shrimp (*Litopenaeus vannamei*). Cryobiology 48(3), 349-356. https://doi.org/https://doi.org/10.1016/j.cryobiol.2004.03.003.

Li, N., Hou, Y.-H., Jing, W.-X., Dahms, H.-U., Wang, L., 2016. Quality decline and oxidative damage in sperm of freshwater crab *Sinopotamon henanense* exposed to lead. Ecotoxicol. Environ. Saf. 130, : 193-198. https://doi.org/https://doi.org/10.1016/j.ecoenv.2016.03.042.

Liu, K., Mao, X., Pan, F., Chen, Y., An, R., 2023. Correlation analysis of sperm DNA fragmentation index with semen parameters and the effect of sperm DFI on outcomes of ART. Sci. Rep. 13(1), 2717. https://doi.org/10.1038/s41598-023-28765-z.

Liu, Q.H., Li, J., Zhang, S.C., Xiao, Z.Z., Ding, F.H., Yu, D.D., Xu, X.Z., 2007. Flow cytometry and ultrastructure of cryopreserved red seabream (*Pagrus major*) sperm. Theriogenology 67(6), 1168-1174. https://doi.org/https://doi.org/10.1016/j.theriogenology.2006.12.013.

López Greco, L.S., Lo Nostro, F.L., 2008. Structural changes in the spermatophore of the freshwater 'red claw' crayfish *Cherax quadricarinatus* (Von Martens, 1898) (Decapoda, Parastacidae). Acta Zool. (Stockholm) 89(2), 149-155. https://doi.org/http://dx.doi.org/10.1111/j.1463-6395.2007.00303.x.

Ma, D., Hou, Y., Du, L., Li, N., Xuan, R., Wang, F., Jing, W., Wang, L., 2013. Oxidative damages and ultrastructural changes in the sperm of freshwater crab *Sinopotamon henanense* exposed to cadmium. Ecotoxicol. Environ. Saf. 98, : 244-249. https://doi.org/https://doi.org/10.1016/j.ecoenv.2013.08.004.

MacDiarmid, A.B., Butler, M.J., 1999. Sperm economy and limitation in spiny lobsters. Behav. Ecol. Sociobiol. 46(1), 14-24.

Marc, A.F., Guppy, J.L., Bauer, P., Mulvey, P., Jerry, D.R., Paris, D.B.B.P., 2021. Validation of advanced tools to evaluate sperm function in barramundi (*Lates calcarifer*). Aquaculture 531(735802), 1-14. https://doi.org/https://doi.org/10.1016/j.aquaculture.2020.735802.

Masser, M.P., Rouse, D.B., 1997. Australian red claw crayfish, Southern Regional Aquaculture Center Publication. United States Department of Agriculture, Cooperative States Research, Education, and Extension Service, United States of America, pp. 1-7.

Medley, P.B.C., Jones, C., W. Jr Avault, J., 1994. A global perspective of the culture of Australian redclaw crayfish, *Cherax quadricarinatus*: Production, economics and marketing. J. World Aquac. Soc. 25(4), 6-13.

Meunpol, O., Meejing, P., Piyatiratitivorakul, S., 2005. Maturation diet based on fatty acid content for male *Penaeus monodon* (Fabricius) broodstock. Aquac. Res. 36(12), 1216-1225. https://doi.org/https://doi.org/10.1111/j.1365-2109.2005.01342.x.

Niksirat, H., Kouba, A., Kozák, P., 2014. Post-mating morphological changes in the spermatozoon and spermatophore wall of the crayfish *Astacus leptodactylus*: Insight into a non-motile spermatozoon. Anim. Reprod. Sci. 149(3), 325-334. https://doi.org/https://doi.org/10.1016/j.anireprosci.2014.07.017.

Niksirat, H., Kouba, A., Pšenička, M., Kuklina, I., Kozák, P., 2013. Ultrastructure of spermatozoa from three genera of crayfish *Orconectes*, *Procambarus* and *Astacus* (Decapoda: Astacoidea): New findings and comparisons. Zool. Anz. 252(2), 226-233. https://doi.org/https://doi.org/10.1016/j.jcz.2012.06.002.

Noor-Hidayati, A.B., Shahreza, M.S., Munafi, A.B.A.-., Ikhwanuddin, M., 2014. Sperm quality assessment of Banana Shrimp *Fenneropenaeus merguiensis* (De Man, 1888) from ultraviolet irradiation for initial development of gynogenesis application. J. Fish. Aquat. Sci. 9(4), 187-196. https://doi.org/https://dx.doi.org/10.3923/jfas.2014.187.196.

Parnes, S., Sagi, A., 2002. Intensification of redclaw crayfish *Cherax quadricarinatus* culture. I. Hatchery and nursery system. Aquac. Eng. 26(4), 251-262. https://doi.org/https://doi.org/10.1016/S0144-8609(02)00034-1.

Peña-Almaraz, O.A., Cortés-Jacinto, E., Vega-Villasante, F., Arcos-Ortega, G.F., Badillo-Zapata, D., Vargas-Ceballos, M.A., 2022. Sperm viability in wild-caught males of *Macrobrachium tenellum* (Smith, 1871)(Decapoda: Caridea: Palaemonidae) fed with different diets. Nauplius 30, 1 - 14.

Peña, S.T., Jr., Gummow, B., Parker, A.J., Paris, D.B.B.P., 2017. Revisiting summer infertility in the pig: could heat stress-induced sperm DNA damage negatively affect early embryo development? Anim. Prod. Sci. 57(10), 1975-1983. https://doi.org/https://doi.org/10.1071/AN16079.

Peña, S.T., Jr., Stone, F., Gummow, B., Parker, A.J., Paris, D.B.B.P., 2019. Tropical summer induces DNA fragmentation in boar spermatozoa: implications for evaluating seasonal infertility. Reprod. Fertil. Dev. 31(3), 590-601. https://doi.org/https://doi.org/10.1071/RD18159.

Pérez-Cerezales, S., Martínez-Páramo, S., Beirão, J., Herráez, M.P., 2010. Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 139(6), 989. https://doi.org/10.1530/rep-10-0037.

Pérez-Cerezales, S., Martínez-Páramo, S., Cabrita, E., Martínez-Pastor, F., de Paz, P., Herráez, M.P., 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71(4), 605-613. https://doi.org/https://doi.org/10.1016/j.theriogenology.2008.09.057.

Pérez-Rodríguez, J.C., Gómez-Gutiérrez, J., López-Greco, L.S., Cortés-Jacinto, E., 2019. Spermatophore production and sperm quality of the river prawn *Macrobrachium americanum* Spence Bate, 1868 fed with different diets. Aquac. Res. 0(0), 1-13. https://doi.org/https://doi.org/10.1111/are.14265.

Perez-Velazquez, M., Bray, W.A., Lawrence, A.L., Gatlin, D.M., Gonzalez-Felix, M.L., 2001. Effect of temperature on sperm quality of captive *Litopenaeus vannamei* broodstock. Aquaculture 198(3), 209-218. https://doi.org/https://doi.org/10.1016/S0044-8486(01)00510-5.

Qiu, J., Wang, W.-N., Wang, L.-j., Liu, Y.-F., Wang, A.-L., 2011. Oxidative stress, DNA damage and osmolality in the Pacific white shrimp, *Litopenaeus vannamei* exposed to acute low temperature stress. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 154(1), 36-41. https://doi.org/https://doi.org/10.1016/j.cbpc.2011.02.007.

R.Core.Team, 2022. R: A language and environment for statistical computing, R Foundation for Statistical Computing. Vienna, Austria.

Rendón Rodríguez, S., Macías Regalado, E., Calderón Pérez, J.A., Núñez Pastén, A., Solís Ibarra, R., 2007. Comparison of some reproductive characteristics of farmed and wild white shrimp males *Litopenaeus vannamei* (Decapoda: Penaeidae). Rev. Biol. Trop. 55(1), 199-206. https://doi.org/10.15517/rbt.v55i1.6071.

Riesco, M.F., Valcarce, D.G., Martínez-Vázquez, J.M., Robles, V., 2019. Effect of low sperm quality on progeny: a study on zebrafish as model species. Sci. Rep. 9(1), 11192. https://doi.org/10.1038/s41598-019-47702-7.

Rigg, D., Seymour, J., Courtney, R., Jones, C., 2020. A review of juvenile redclaw crayfish *Cherax quadricarinatus* (von Martens, 1898) production in aquaculture. Freshw. Crayfish 25(1), 13-30. https://doi.org/10.5869/fc.2020.v25-1.013.

Rijsselaere, T., Maes, D., Hoflack, G., De Kruif, A., Van Soom, A., 2007. Effect of body weight, age and breeding history on canine sperm quality parameters measured by the Hamilton-Thorne analyser. Reprod. Domest. Anim. 42(2), 143-148. https://doi.org/https://doi.org/10.1111/j.1439-0531.2006.00743.x.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234(1), 1-28. https://doi.org/https://doi.org/10.1016/j.aquaculture.2003.12.006.

Samuel, M.J., Kannupandi, T., Soundarapandian, P., 1999. Nutritional effects on male reproductive performance in the freshwater prawn *Macrobrachium malcolmsonii* (H. Milne Edwards). Aquaculture 172(3), 327-333. https://doi.org/https://doi.org/10.1016/S0044-8486(98)00500-6.

Sato, T., Ashidate, M., Goshima, S., 2004. A new method to extract sperm from spermatophores of the male spiny king crab *Paralithodes brevipes* Anomura Lithodidae. Crust. Res. 3(3), 10-14. https://doi.org/10.18353/crustacea.33.0_10.

Sato, T., Ashidate, M., Wada, S., Goshima, S., 2005. Effects of male mating frequency and male size on ejaculate size and reproductive success of female spiny crab *Paralithodes brevipes*. Mar. Ecol.-Progr Ser. 296, 251-262. https://doi.org/10.3354/meps296251.

Sato, T., Yoseda, K., Abe, O., Shibuno, T., 2008. Male maturity, number of sperm, and spermatophore size relationships in the coconut crab *Birgus Latro* on Hatoma Island, Southern Japan. J. Crust. Biol. 28(4), 663-668. https://doi.org/https://doi.org/10.1651/07-2966.1.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9(7), 676 - 682. https://doi.org/10.1038/nmeth.2019.

Sheikh, H., Yunus, K., John, A., Kassim, Z., Ichwan, S., 2019. Spermiogram of wild and captive Malaysian Horseshoe Crab (*Tachypleus gigas*) from Pantai Balok, Kuantan, Pahang, Malaysia. Sains Malays. 48, 325-328. https://doi.org/10.17576/jsm-2019-4802-08.

Shu, L., Zhang, X., 2017. Shrimp miR-12 Suppresses White Spot Syndrome Virus infection by synchronously triggering antiviral phagocytosis and apoptosis pathways. Front. immunol. 8(855), 1-13. https://doi.org/https://doi.org/10.3389/fimmu.2017.00855.

Silva, E.F., Batista, A.M., Silva, S.V., Calazans, N., Castelo Branco, T., Guerra, M.M.P., Peixoto, S., 2015. Use of fluorescent microscopy for sperm quality of penaeids. J. Crust. Biol. 35(1), 26-29. https://doi.org/https://doi.org/10.1163/1937240X-00002295.

Simon, L., Murphy, K., Shamsi, M.B., Liu, L., Emery, B., Aston, K.I., Hotaling, J., Carrell, D.T., 2014. Paternal influence of sperm DNA integrity on early embryonic development. Hum. Reprod. 29(11), 2402-2412. https://doi.org/https://doi.org/10.1093/humrep/deu228.

Stevenson, J., Jerry, D., Owens, L., 2013. Redcla selective breeding project, Rural Industries Research and Development Corporation. Rural Industries Research and Development Corporation, Rural Industries Research and Development Corporation Level 2, 15 National Circuit BARTON ACT 2600 p. 58.

Subramoniam, T., 2017a. Chapter 11 - Spermatogenesis, in: Subramoniam, T. (Ed.), Sexual Biology and Reproduction in Crustaceans. Academic Press, Elsevier Inc., pp. 293-324.

Subramoniam, T., 2017b. Spermatophore and Sperm Transfer Mechanisms, in: Sexual Biology and Reproduction in Crustaceans. Academic Press, Elsevier Inc., pp. 325-368.

Sugiani, D., Lusiastuti, A.M., Taukhid, nbsp, Purwaningsih, U., 2015. Treatments for *Temnocephalids Ectosymbiont Craspedella sp.* on *Cherax quadricarinatus* and *Cherax albertisii* (Papua Freshwater Lobster). World J. Eng. Tech. 3(4), 48 - 54. https://doi.org/10.4236/wjet.2015.34C006.

Swinscow, T.D.V., 1997. Correlation and regression, in: Campbell, M.J. (Ed.), Statistics at square one (9th edition). BMJ Publishing Group, University of Southampton.

Tesi, M., Sabatini, C., Vannozzi, I., Di Petta, G., Panzani, D., Camillo, F., Rota, A., 2018. Variables affecting semen quality and its relation to fertility in the dog: A retrospective study. Theriogenology 118, 34-39. https://doi.org/https://doi.org/10.1016/j.theriogenology.2018.05.018.

Turri, F., Madeddu, M., Gliozzi, T.M., Gandini, G., Pizzi, F., 2016. Relationship between body weight, sexual secondary traits and epididymal semen quality in the Alpine goat. Small Rumin. Res. 135, 81-84. https://doi.org/https://doi.org/10.1016/j.smallrumres.2015.12.017.

Van den Berghe, F., Paris, M.C.J., Briggs, M.B., Farstad, W.K., Paris, D.B.B.P., 2018. A two-step dilution tris-egg yolk extender containing Equex STM significantly improves sperm cryopreservation in the African wild dog (*Lycaon pictus*). Cryobiology 80, 18-25. https://doi.org/https://doi.org/10.1016/j.cryobiol.2017.12.095.

Vuthiphandchai, V., Nimrat, S., Kotcharat, S., Bart, A.N., 2007. Development of a cryopreservation protocol for long-term storage of black tiger shrimp (*Penaeus*

monodon) spermatophores. Theriogenology 68(8), 1192-1199. https://doi.org/https://doi.org/10.1016/j.theriogenology.2007.08.024.

Wang, E.Y., Huang, Y., Du, Q.Y., Yao, G.D., Sun, Y.P., 2017. Body mass index effects sperm quality: a retrospective study in Northern China. Asian J. Androl. 19(2), 234-237. https://doi.org/10.4103/1008-682x.169996.

Wang, Y.-L., Sun, W.-J., He, L., Li, Q., Wang, Q., 2015. Morphological alterations of all stages of spermatogenesis and acrosome reaction in Chinese mitten crab *Eriocheir sinensis*. Cell Tissue Res. 360(2), 401-412. https://doi.org/https://doi.org/10.1007/s00441-014-2092-5.

WHO, 2021. WHO laboratory manual for the examination and processing of human semen, 6th edition. Sexual and Reproductive Health and Research. Licence: CC BY-NC-SA 3.0 IGO, Geneva: World Health Organization.

Xu, X.H., Xu, X.H., Yan, B.L., Xu, X.H., Yan, B.L., Peng, D.S., 2014. Comparative study on the acrosin activity in sperm of the marine crab *Charybdis japonica* (A. Milne-Edwards, 1861) (Brachyura, Portunidae) under different environmental conditions. Crustaceana 87(13), 1473-1485. https://doi.org/https://doi.org/10.1163/15685403-00003369.

Yang, H., Li, G., Jin, H., Guo, Y., Sun, Y., 2019. The effect of sperm DNA fragmentation index on assisted reproductive technology outcomes and its relationship with semen parameters and lifestyle. Transl. Androl. Urol. 8(4), 356-365.

Zheng, W.-W., Song, G., Wang, Q.-L., Liu, S.-W., Zhu, X.-L., Deng, S.-M., Zhong, A., Tan, Y.-M., Tan, Y., 2018. Sperm DNA damage has a negative effect on early embryonic development following *in vitro* fertilization. Asian J. Androl. 20(1), 75-79. https://doi.org/https://dx.doi.org/10.4103%2Faja.aja_19_17.

Chapter 4. Quality of spermatozoa extracted from spermatophore removed by dissection or extruded by electroejaculation from wild-caught redclaw crayfish (*Cherax quadricarinatus*)

4.1. Abstract

In this study, we determined the efficiency of electroejaculation and assessed sperm quality using conventional and advanced reproductive biomarkers following the extraction of spermatophores by either dissection or electroejaculation from wild-caught redclaw crayfish. In Experiment 1, sexually mature male crayfish (> 40 g) were collected from Ross River Dam, northern Queensland and were subjected to electroejaculation to evaluate the efficiency of electrical stimulation to extrude spermatophores. In Experiment 2, 33 male crayfish weighing 99.4 ± 7.6 g (mean ± SEM) were subjected to sperm quality assessment following isolation from spermatophores collected by dissection and electroejaculation. The odds of extracting spermatophore were affected by body weights ($p = 0.029$) and were greatest within crayfish weighing between 60 and 130 g. The success rate in extracting spermatophores from either gonopore using electroejaculation was 22.2 % (n = 153). The mean weight of spermatophores from left (0.016 ± 0.002 g) or right (0.011 ± 0.002 g) gonopores were comparable and were not significantly influenced by body weight ($p = 0.430$). A greater mean concentration of spermatozoa, sperm viability, total potential fertile sperm concentration (TPFSC) and lesser sperm DNA fragmentation were measured following the extraction of spermatophores by dissection, than those by electroejaculation ($p < 0.001$). Together, these data suggest that the method used for spermatophore collection impacts sperm quality in redclaw crayfish. Spermatophores collected following dissection provided better sperm quality and are recommended for developing advanced breeding techniques, including sperm freezing and artificial reproduction techniques. However, electroejaculation

is a simple, non-lethal alternative for collecting spermatophores for routine breeding and commercial production of redclaw.

Keywords: Aquaculture, crustaceans, DNA fragmentation, fertility, spermatophore, sperm quality

4.2. Introduction

Redclaw crayfish are relatively large freshwater decapods that are fast-growing, non-burrowing, and can withstand crowding and a wide range of environmental conditions (Jones and Valverde, 2020; Rigg et al., 2020; Haubrock et al., 2021). Average meat recovery for redclaw crayfish is 30 % body weight, which is relatively higher than other farmed *Cherax* species (12 – 26 %; Bitomsky, 2008), with taste highly comparable to other crustaceans available in the global seafood market (Medley et al., 1994; Masser and Rouse, 1997; Jones, 2011; Ghanawi and Saoud, 2012). Redclaw crayfish are also farmed in Southeast Asia, Central and South America and the USA. While most products are predominantly marketed locally in each region, the export potential is significant (Irvin et al., 2018; Mobsby, 2018; FAO-FIGIS, 2019).

Redclaw farming has been advocated for extensive farming in northern Australia (Irvin et al., 2018) and has considerable potential for growth in aquaculture. However, despite developments in breeding technology since the 1990s, production has been relatively low compared to other aquaculture species (Jones et al., 1996; Jones and Valverde, 2020; Rigg et al., 2020). Production challenges include insufficient juvenile supply and a limited production season restricted mainly to summer (Jones et al., 1996; Jones, 2011; FAO-FIGIS, 2019; Rigg

et al., 2020). The traditional method of stocking adult broodstock in ponds for the natural production of juvenile redclaw has yielded poor survival rates (5.0 – 10.0 %), variability in quality, and high rates of cannibalism (Jones, 1995; Masser and Rouse, 1997; Jones, 2011; Irvin et al., 2018; Jones and Valverde, 2020). Additionally, the absence of large numbers of intensively produced juveniles for stocking in grow-out ponds exacerbates the production shortage, leading to an unstable supply of seedstock for redclaw farmers. To address these major challenges, further refinements to juvenile production strategies are required to achieve consistent commercial production of craylings for both market supply and to facilitate industry expansion (Yeh and Rouse, 1994; Parnes and Sagi, 2002; Jones and Valverde, 2020; Rigg et al., 2020).

The influence of broodstock fertility on reproductive efficiency and the production of redclaw juveniles has not been investigated previously. The success of producing a normal embryo and survival depends on the quality of eggs and sperm cells (Bobe and Labbé, 2010). When either or both gametes are of poor quality, it can lead to low fertilisation, hatching rates, and survival amongst juveniles (Bobe and Labbé, 2010; Riesco et al., 2019). In crustaceans, fecundity, spawning and hatching rates have been the traditional basis for addressing egg quality (King, 1993; Yeh and Rouse, 1994; Carmona-Osalde et al., 2004; Rodriguez-Gonzalez et al., 2006; Li et al., 2007; Rodríguez-González et al., 2009; Rodríguez-González et al., 2011; Liu et al., 2013), but little attention has been given to the role of sperm quality above fertilisation. There is growing evidence that sperm quality impacts the early development and survival of fish larvae (Bobe and Labbé, 2010; Migaud et al., 2013; Kowalski and Cejko, 2019; Riesco et al., 2019). The paternal genetic contribution correlates significantly to morphological embryonic deformities, lower offspring survival in finfishes, and negatively impacts production (Pérez-Cerezales et al., 2010; Devaux et al., 2011; Riesco

et al., 2019). In redclaw crayfish, one study has examined sperm quality for breeding (Bugnot and López Greco, 2009), while others investigated sperm morphology (Beach and Talbot, 1987; Kouba et al., 2015). The first step to understanding the impact of sperm quality on fertilisation, egg, and larval development is to develop robust diagnostic measures. Therefore, the availability of advanced sperm quality biomarkers for redclaw crayfish would be an invaluable tool to improve sperm quality estimation and better understand its contribution to successful embryonic development (Bobe and Labbé, 2010; Cabrita et al., 2014).

The initial step in assessing sperm quality in decapod crustaceans involves the extraction of spermatophores (Beirão et al., 2019; Aquino et al., 2022). The most common method is the dissection of the vas deferens to remove spermatophores, which involves sacrificing male broodstock and suspending the tissue in crayfish saline solution to enable subsequent assessment of sperm quality (Bugnot and López Greco, 2009). This terminal procedure, however, prevents the use of male broodstock for future breeding and production. Non-lethal alternatives that allow the use of male broodstock for future breeding and production include manual stripping (Bart et al., 2006; Castelo-Branco et al., 2015), and electroejaculation (Sandifer et al., 1984; Jerry, 2001). While manual stripping is commonly used in decapod crustaceans, specifically in *penaeids*, the success of spermatophore extrusion varies with the handler's experience and the decapod crustacean species. Notably, manual stripping is difficult for hard-shelled decapod crustaceans such as redclaw, leaving electroejaculation as the only non-lethal alternative (Kooda-Cisco and Talbot, 1983; Jerry, 2001; Farhadi et al., 2019a). Electroejaculation involves the application of a pair of electrodes connected to an AC variable transformer to the base of the abdominal keel before the fifth pereiopods to induce muscle contractions, resulting in spermatophore extrusion (Jerry, 2001; Diggles, 2019). The use of electroejaculation to extrude spermatophore has been successfully applied to some

species of freshwater crayfish including narrow-clawed (*Pontastacus leptodactylus*; Niksirat et al., 2014b; Farhadi et al., 2019a), noble (*Astacus astacus*; Niksirat et al., 2015), signal (*Pacifastacus leniusculus*; Niksirat et al., 2014a; Niksirat et al., 2016), yabby (*Cherax destructor*; Jerry, 2001), and redclaw (*C. quadricarinatus*; Kouba et al., 2015). The development of a spermatophore extraction protocol for redclaw hatcheries is desirable for the potential establishment of intensive breeding techniques, including sperm cryopreservation and artificial fertilisation.

Reports on decapod crustacean sperm quality obtained using various spermatophore collection techniques have been limited (Nakayama et al., 2008; Farhadi et al., 2019a). The feasibility of the development and optimisation of conventional and advanced sperm quality diagnostic tools for quantifying sperm quality in redclaw crayfish has been reported recently (Aquino et al., 2023, Chapter 3). To date, no previous studies have investigated the utilisation of electroejaculation for spermatophore collection in redclaw nor directly compared the number and quality of spermatozoa extracted using either electroejaculation or dissection. Therefore, the purpose of this study was two-fold: to evaluate the efficiency of spermatophore collection by electroejaculation and to compare sperm quality following spermatophore collection by dissection and electroejaculation using conventional and advanced diagnostic tools in redclaw crayfish.

4.3. Material and Methods

4.3.1. Experimental animals

Sexually mature male redclaw crayfish were collected using baited funnel traps (Jones, 1990) from the Ross River Dam, Townsville, Queensland (19.4090° S, 146.7348° E) during the summer from November 2020 to March 2021 for Experiment 1 (n = 153), and from January to February 2022 for Experiment 2 (n = 33). Animals were transferred to the Australian Crayfish Hatchery (ACH, Townsville, Queensland) recirculating facility in a 500-L tank containing approximately 250-L dechlorinated freshwater water until sampled. The Institutional Animal Ethics Committee deemed crustaceans exempt from animal ethics requirements, negating the need for ethics approval. This study conforms to ACH protocols for ethical animal treatment and industry best practices.

4.3.2. Experiment 1: Electroejaculation efficiency

On the day of collection, animals at the intermolt stage and weighing > 40 g were anaesthetised in chilled freshwater (15 °C) for 10 min, weighed individually, and had their gonopores swabbed with 70 % ethanol to eliminate surface bacteria. Electroejaculation and preparation of single-sperm cell suspension was performed according to the methods described in Chapter 3.

Electroejaculation was conducted randomly on both the left and right gonopores. The successful response of males to electroejaculation was recorded when complete extrusion of spermatophores occurred on either side of the gonopore. The efficiency of electroejaculation

was defined as the number of individuals that extruded spermatophores, divided by the total number of animals electroejaculation was attempted on and expressed as a percentage.

4.3.3. Experiment 2: Sperm quality evaluation from spermatophores collected by electroejaculation and dissection

In Experiment 2, crayfish > 50 g were subjected to spermatophore collection since results from Experiment 1 suggested that the probability of collecting spermatophores from crayfish < 60 g was low (< 10 %). In addition, since 68 % of the electroejaculated spermatophores were extruded from the left compared to the right gonopore in Experiment 1, the collection of spermatophores was attempted first on the left gonopore. If electroejaculation applied to the left gonopore failed to extrude the spermatophore after ten stimulation cycles, collection of spermatophore from the right gonopore was attempted. Collection of spermatophore by dissection was performed following the method described by Bugnot and López Greco (2009) and only on each animal that extruded a spermatophore immediately following electroejaculation (n = 33). Briefly, a section (one cm) from the distal vas deferens (DVD) was obtained. The spermatophore was squeezed gently from the DVD using a fine tweezer directly into a tube with 1 ml Ca^{2+}- free saline (CFS) solution. Only animals with complete appendages (*i.e.*, chelipeds and limbs) and unmelanized gonopores were subjected to collection of spermatophores. After collection, spermatophores were processed, and spermatozoa were extracted using a protocol similar to Experiment 1. Aliquots were transferred to new tubes for sperm viability and DNA fragmentation assays.

4.3.3.a. Sperm concentration

The concentration of spermatozoa in each spermatophore was estimated using the protocol described in Chapter 3.

4.3.3.b. Sperm viability

To assess sperm viability in redclaw, the protocol outlined in Chapter 3 was followed. Briefly, the integrity of the plasma membrane in spermatozoa was assessed using Hoechst 33342 (H_{342}; Sigma-Aldrich Pty. Ltd., Macquarie Park NSW, Australia) and propidium iodide (PI; Sigma-Aldrich Pty. Ltd., Macquarie Park NSW, Australia) nuclear staining. The quantification of viable spermatozoa expressed in percentage was performed using flow cytometry.

4.3.3.c. DNA fragmentation assay

The extent of DNA fragmentation was evaluated through the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method with slight modifications from the manufacturer's protocol (*In Situ* Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Mannheim, Germany) and following the methods in Chapter 3. The quantification of DNA-damaged spermatozoa expressed in percentage was carried out using flow cytometry.

4.3.3.d. Flow cytometry

The flow cytometry analysis was performed following the methods in Chapter 3.

4.3.3.e. Determination of total potential fertile sperm concentration (TPFSC)

The TPFSC was calculated using the formula described in Chapter 3.

4.3.4. Statistical analysis

Data analysis and graphing were conducted using custom scripts in RStudio version 4.1.2 (RStudio Inc., Boston, MA, USA; R Core Team, 2022). The odds of collecting spermatophores by electroejaculation were assessed using logistic regression with body weight included as a covariate, while the relationship between the side of extruding gonopores and body and spermatophore weights was evaluated using multiple linear regression with interaction effects. The quality of sperm isolated from spermatophores extruded by electroejaculation and removed by dissection was evaluated using a paired *t*-test when assumptions were met; otherwise, the Wilcoxon Signed-Rank test was employed. The Shapiro-Wilk test assessed the normal distribution of data residuals from the regression analysis and data differences for the Paired T-test. The homoscedasticity of data residuals in regression analysis was evaluated using the Studentized Breusch-Pagan test. The *wilcox_effsize (r)* function in RStudio determined the effect size needed when reporting Wilcoxon Signed-Rank test results. A statistical significance in all analyses relied on $p \leq 0.05$. All data were displayed as mean ± SEM.

4.4. Results

4.4.1. Experiment 1: Electroejaculation efficiency

The probability of extracting spermatophore (p) was estimated by fitting the logistic equation $p = 1/(1 + e^{-(-0.00068 (BW^2) + 0.12913 (BW) - 6.9785)})$, where BW is the body weight of redclaw crayfish (z = -2.19, *p = 0.029*, 95 % CI [0.9987 – 0.9999]). Analysis of deviance of the logistic regression showed that the effect of body weight required a quadratic term allowing the effect of body weight to decrease at the higher body weights (X^2 (2, N = 153) = 6.11, *p = 0.047*; Figure 4.1). Out of 153 redclaw males, 22.2 % extruded spermatophores completely by electroejaculation. The mean weight of the extruded spermatophore did not differ between the side of extrusion (0.016 ± 0.02 g *versus* 0.011 ± 0.02 g for the left and right sides, respectively; *p = 0.430*) and was not significantly affected by body weight (*p = 0.272*), nor the interaction between the side of extrusion and body weight (*p = 0.799*). No significant linear relationship was found between spermatophore weight from either the left or right gonopores and body weight (R^2 = 0.16, F (3, 28) = 1.749, *p = 0.180).*

4.4.2. Comparing sperm quality between spermatophore collection methods

The mean sperm concentration and TPFSC were significantly greater following the collection of spermatophores by dissection than those collected by electroejaculation (Table 4.1). Median sperm viability was compared using the Wilcoxon signed rank test due to violation of assumptions of normality. Using this test, median sperm viability was found to be significantly greater in spermatozoa extracted from spermatophores collected by dissection than those collected by electroejaculation (81.4 %, CI: 27.7 – 98.8 % *versus* 64.4%, 95 % CI:

24.8 – 90.3 %; V = 508, r = 0.71; $p < 0.001$). The mean percentage of spermatozoa exhibiting DNA fragmentation was significantly greater in spermatozoa extracted from spermatophores collected by electroejaculation than those collected by dissection (Table 4.1).

Table 4.1. Comparison of sperm quality (mean ± SEM) from spermatophores collected by dissection and electroejaculation from wild-caught redclaw crayfish using a paired T-test analysis at $p \leq 0.05$ statistical significance (*Cherax quadricarinatus*).

Sperm quality indicators	Methods of spermatophore collection		*p-value*
	Dissection	Electroejaculation	
Sperm concentration (cells/ml)	$85.0 \times 10^4 \pm 8.5 \times 10^{4\,b}$	$44 \times 10^4 \pm 5.4 \times 10^{4\,a}$	< 0.001
TPFSC (cells/ml)	$56 \times 10^4 \pm 6.2 \times 10^{4\,b}$	$24 \times 10^4 \pm 3.6 \times 10^{4\,a}$	< 0.001
Sperm DNA fragmentation (%)	14.0 ± 2.3^{a}	19.4 ± 2.6^{b}	*0.014*

^{ab} Different superscript letters within rows indicate significant differences between the two collection methods. TPFSC - total potential fertile sperm concentration.

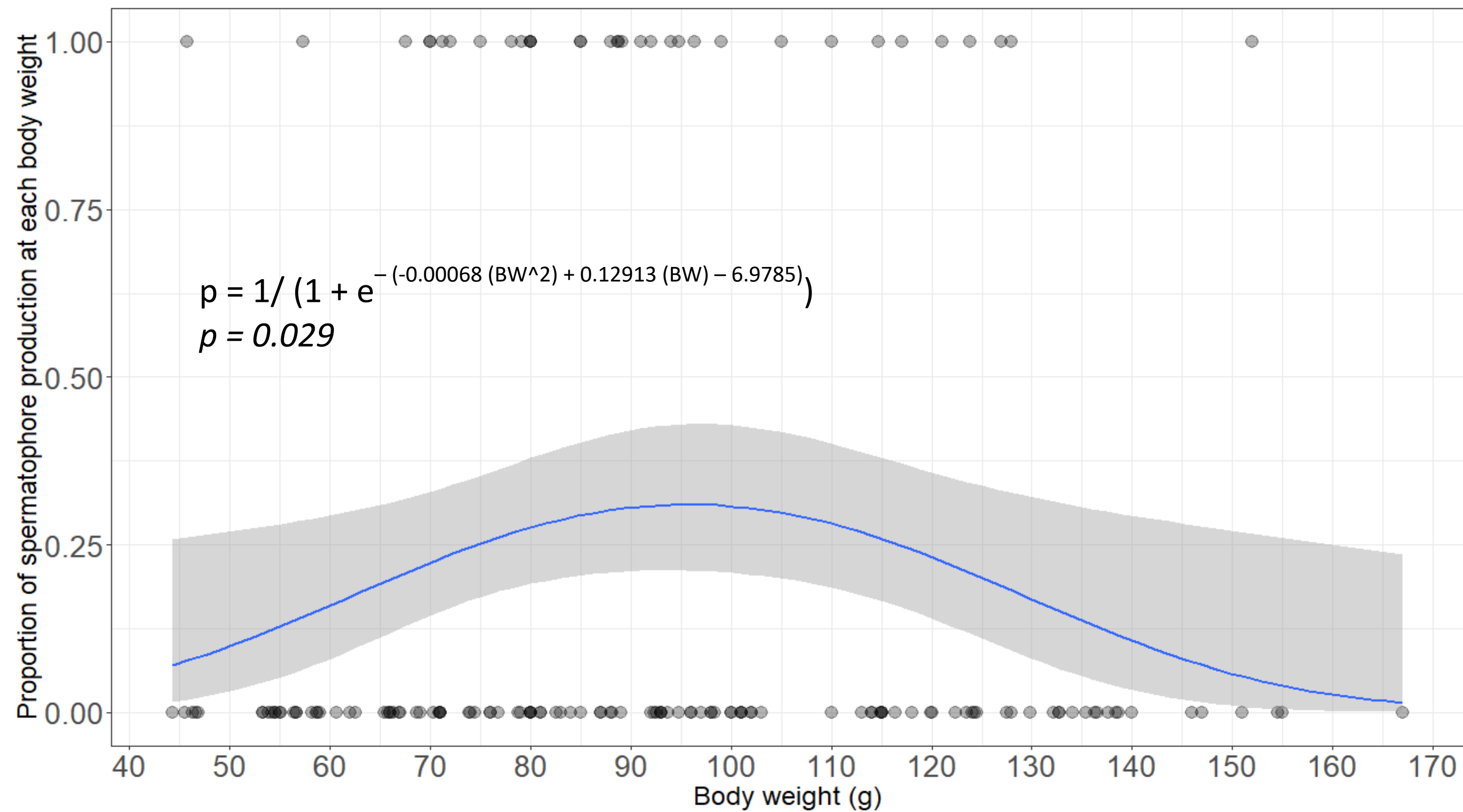

Figure 4.1. The model prediction of spermatophore extrusion across body weights with a 95 % confidence interval (grey band) in wild-caught redclaw crayfish (*Cherax quadricarinatus*). The circles aligned with 1.00 represent redclaw males that produced spermatophores, while those aligned with 0.00 did not. The blue line indicates the proportion of spermatophore production based on the body weight. Logistic regression is significant at $p \leq 0.05$. p, probability of extracting spermatophore.

4.5. Discussion

This study investigated the efficiency of electroejaculation in collecting spermatophores from wild-caught redclaw crayfish. In addition, the quality of spermatozoa extracted from spermatophores removed by dissection was compared with those extruded by electroejaculation. A standout finding was that the method of spermatophore collection significantly influences sperm quality in redclaw crayfish. Dissection removal of spermatophores resulted in 38.6 % lower sperm DNA fragmentation and over double the concentration of potentially fertile spermatozoa compared to electroejaculation-extracted spermatophores. These data have implications for redclaw breeding strategies, particularly when evaluating and utilising male gametes for sperm banking or artificial reproduction.

In Experiment 1, spermatophores were successfully extruded following electroejaculation from wild-caught male redclaw crayfish across a wide range of body weights. The predicted model illustrated in Figure 4.1 showed a quadratic trend, where the proportion of crayfish with body weights between 60 and 130 g yielded spermatophore extrusion rates of 15 to 30 % following ejaculation. This suggests that electroejaculation can efficiently produce spermatophores from male redclaw crayfish within a specific weight range, though beyond this range, the likelihood of spermatophore collection is reduced. While the success rate of extruding redclaw spermatophore completely from single gonopore using electroejaculation was only 22.2 % (n = 153), it is comparable to rates reported by Jerry (2001) in yabbies from the same genus as redclaw (*Cherax destructor*; 19.0 %, n = 100), and those reported in *Penaeus* spp (26.9 %, n = 78; Sandifer et al., 1984). Similarly, Farhadi et al. (2019a) reported that small narrow-clawed crayfish (*Pontascus leptodactylus*) with a carapace length of < 6.5 cm yielded fewer spermatophore using electroejaculation than larger males with

carapace lengths of > 6.5 cm. These authors hypothesised that the inferior ejaculate capacity of smaller crayfish was due to fewer spermatozoa held in the vas deferens (Farhadi et al., 2019a; Farhadi et al., 2019b). In decapod crustaceans, larger decapod males have greater sperm reserves in the vas deferens, allowing them to produce more spermatophores (Sainte-Marie, 2007). The results of this study, however, demonstrated that the success rate of extracting spermatophore in wild-caught redclaw crayfish peaked between 60 to 130 g total body weights and declined outside of this range. The decline in the percentage of spermatophore extracted after electroejaculation of male crayfish heavier than 130 g is possibly attributed to age-related degeneration of reproductive performance. Senescence of males' reproductive performance was also demonstrated by Rubolini et al. (2007), where spermatophore size decreased with increasing body size, with larger, older males exhibiting difficulties with mating and spermatophore production as evidenced by fewer fertilised females in a breeding trial for freshwater crayfish (*Austropotamobius italicus*). Notably, the range of crayfish body weight for optimal spermatophore recovery in the current study reflects the range in size of male breeders used at the Australian Crayfish Hatchery for redclaw breeding.

In wild-caught redclaw crayfish, the spermatophore weight correlates positively with sperm concentration and TPFSC but inversely with sperm DNA fragmentation and, as such, could be used as a preliminary indicator of sperm quality (Aquino et al., 2023, Chapter 3). In this study, spermatophore weights collected by electroejaculation were comparable between left and right gonopores, which is consistent with the findings of Rendón Rodríguez et al. (2007) following manual extrusion of spermatophores from wild-caught male whiteleg shrimps (*Litopenaeus vannamei*). No significant association between total body and spermatophore weight was found in this study, which is also similar to the results reported in other

freshwater crustaceans (Aquino et al., 2023, Chapter 3; Peña-Almaraz et al., 2022). These findings suggest that once redclaw males have reached sexual maturity, larger body weights may not confer any advantage to obtaining heavier spermatophores. Whether sperm quality is comparable from left and right gonopores following electroejaculation also requires further validation.

This study demonstrated that electroejaculation is a reliable and non-lethal method of collecting spermatophores from male redclaw crayfish. Jerry (2001) reported that electroejaculation has no adverse side effects on male crayfish broodstock in the short term. It is a simple, fast (3 – 5 min) and economical method of collecting spermatophores (Kubec et al., 2012; Farhadi et al., 2019a). Using electroejaculation, male crayfish broodstock can be pre-screened and maintained in the hatchery for potential collection of spermatophore on multiple occasions, although further study is needed to determine the effects of repeated collection. Repeated collection of spermatophores with electroejaculation may enable the long-term storage of spermatozoa in sperm banks and facilitate the development and use of artificial fertilisation techniques (Jerry, 2001; Nakayama et al., 2008; Farhadi et al., 2019a).

In Experiment 2, greater sperm concentration, viability, total potential fertile sperm concentration and reduced DNA fragmentation were obtained in spermatozoa isolated from spermatophores collected by dissection compared to electroejaculation. In narrow-clawed crayfish (*Pontastacus leptodactylus*), Farhadi et al. (2019a) reported no significant difference in sperm viability between spermatophores collected via dissection or electroejaculation when assessed with eosin-nigrosin staining and light microscopy. In other crayfish species, the viability of spermatozoa isolated from spermatophores collected by dissection from white-clawed crayfish (*Austropotamobius italicus;* Galeotti et al., 2012) was 68.0 ± 2.4 %,

which is slightly less than the value of 75.9 ± 3.1% found in this study. The microscopic assessment technique employed by Farhadi et al. (2019a) and Galeotti et al. (2012) relies solely on subjective, visible differences in sperm morphology. In contrast, in Experiment 2, nuclear staining of spermatozoa was assessed with flow cytometry, with 10,000 spermatozoa analysed, which offers a more accurate indicator of sperm viability. In addition, animals in this study were heavier (99.4 ± 7.6 g) than those used by Farhadi et al. (2019a; 59.8 ± 3.1 g) and Galeotti et al. (2012; 34.0 ± 0.8 g). Interestingly, while Farhadi et al. (2019a) reported no difference in sperm viability, the spawning rate of inseminated female crayfish differed significantly between spermatophore collection methods. Using spermatophores obtained from the dissected males, spawning rates of 100 % were achieved by inseminated female narrow-clawed crayfish, compared to a complete absence of spawning following insemination using electroejaculated spermatophores. This supports the findings of Experiment 2 and suggests that compared to electroejaculation, viable sperm yields and improved fertilisation rates may be higher using dissection for the removal of spermatophores. This requires further validation in redclaw crayfish.

Few studies have reported sperm DNA fragmentation in decapod crustaceans, with results varying between species. The extent of spermatozoa DNA fragmentation (17.2 ± 2.5 %) following extrusion of spermatophores by electroejaculation from wild-caught redclaw crayfish in Chapter 3 is comparable to the finding in the current study. In both studies, mean sperm DNA fragmentation values were greater using electroejaculation than those collected by dissection, suggesting electroejaculation may have a detrimental effect on DNA integrity. González-Marín et al. (2012) categorised the causes of DNA fragmentation in animals and humans as those arising from intrinsic or extrinsic factors. Intrinsic factors influencing sperm DNA fragmentation include apoptosis, recombination deficiencies, protamine imbalances or

oxidative stress; while extrinsic factors include handling conditions, time after ejaculation, seminal extenders, storage temperatures, infections and reaction to chemicals or post-testicular oxidative stress (González-Marín et al., 2012). In the current study, it is unlikely that extrinsic factors contributed to elevated sperm DNA fragmentation in the electroejaculation group, compared to the dissection group, given that an identical processing methodology was applied. While it is possible that intrinsic factors, such as increased oxidative stress induced by electroejaculation, contributed to higher levels of DNA fragmentation, the mechanism responsible remains to be elucidated. In vertebrates, there is a noticeable link between oxidative stress and sperm DNA fragmentation (Iommiello et al., 2015; Anel-López et al., 2016). Furthermore, the process of electroejaculation has been found to increase oxidative stress (Fidan et al., 2018) and sperm DNA fragmentation (Restelli et al., 2009; Jiménez-Rabadán et al., 2012). Using the sperm chromatin dispersion test, DNA fragmentation in wild-caught black tiger prawns spermatozoa (*Penaeus monodon*) was reportedly 3.3 ± 1.5 % (mean ± SD; n = 10) following collection via electroejaculation (Feng et al., 2018), levels much lower than this study found. In Experiment 2, the sperm DNA fragmentation was, however, consistent with those of Duangjai et al. (2023), who reported a wide range of DNA-damaged spermatozoa (8 – 32 %) using the Comet assay following spermatophore collection by manual stripping in whiteleg shrimp (*Litopenaeus vannamei*) from four different commercial farms. Further investigation is required to determine if inherent differences exist between crustacean species, if other variables contribute to these apparent differences, or if electroejaculation consistently increases the percentage of DNA-fragmented spermatozoa across species.

Reproductive performance is an important secondary welfare indicator in vertebrates (Grimard et al., 2019). In this study, the physiological stress associated with

electroejaculation during spermatophore collection may cause a reduction in sperm quality. In other crustaceans, the effect of electroejaculation on spermatophore and sperm quality was documented. Leung-Trujillo and Lawrence (1987) and Pérez-Rodríguez et al. (2019) collected spermatophores by electroejaculation in shrimps (*Penaeus setiferus*; weekly for 7 weeks) and freshwater prawns (*Macrobrachium americanum*; every 24 days for 244 days), respectively, and have reported that spermatophore production and sperm quality significantly declined after 20 to 30 days. Similarly, Nakayama et al. (2008) have reported a decline in the weight of spermatophores and sperm count in wild-caught pink shrimps (*Farfantepenaeus paulensis*) following two collections, using electroejaculation 43 days apart. The authors attributed the decline in the weight of spermatophore and sperm count to physiological stress associated with electroejaculation (Nakayama et al., 2008). Studies have recommended that a mix of fresh wet food and a high-quality commercial diet for crustaceans can be used to reduce the effects of natural spermatophore degeneration (production and weight) and may improve sperm quality in decapod crustaceans (Chamberlain and Lawrence, 1981; Samuel et al., 1999; Braga et al., 2010). The results of this study suggest that the method of spermatophore collection also contributes to the sperm quality differences. Further work is warranted to determine if managerial, environmental or genetic factors can be altered to minimise potential adverse effects of electroejaculation on sperm quality in redclaw crayfish.

Although electroejaculation can be effective in decapod crustaceans, it is important to exercise caution when using this method. A strong electric current or frequent stimulus could result in severe damage to the gonopore tissue, and, in some cases, death has been recorded in other species of crustaceans (Nakayama et al., 2008; Diggles, 2019). In the present study, no mortalities were observed within two weeks of electroejaculation. When fertile male

broodstock dies unexpectedly, dissection is the only viable method for recovering male gametes (Koteeswaran and Pandian, 2002). In other crustaceans, cadaveric shrimps have been dissected to recover spermatophore for cryopreservation and subsequent use of extracted viable spermatozoa for artificial fertilisation (Bambozzi et al., 2014; Castelo-Branco et al., 2016; Selvakumar et al., 2018). To date, there are no reports on sperm cryopreservation in redclaw crayfish. An improved understanding of which collection techniques provide superior sperm quality will be beneficial in developing strategies to cryopreserve genetic material from redclaw. This is particularly pertinent for critically endangered species such as *Cherax tenuimanus* (Austin & Bunn, 2010; Duffy et al., 2014), where the number of animals is limited for breeding and stock enhancement programmes.

Several limitations are acknowledged in this study. Other studies conducted in male decapod crustaceans suggest that the production of spermatophore and sperm quality vary between wild-caught and captive animals thus, the potential effects of captivity on the quantity and quality of sperm extracted using electroejaculation and dissection requires further investigation (Pratoomchaat et al., 1993; Rendón Rodríguez et al., 2007; Anand et al., 2023). Although the capacity of male decapod crustaceans to extrude spermatophore following electroejaculation could be an indicator of sexual maturity, it does not guarantee that males are reproductively competent (Goñi et al., 2003; Sato et al., 2008). Sexual maturity of male decapod crustaceans can only be assessed by fertility tests set in a breeding trial, although quantifying sperm quality provides information on the fertilising capacity of male decapods (Cabrita et al., 2009). Electroejaculation has been applied successfully to extrude spermatophores safely in other freshwater crayfish (Jerry, 2001; Niksirat et al., 2014b; Niksirat et al., 2015; Farhadi et al., 2019a) and other decapod crustaceans (Kooda-Cisco and Talbot, 1983; Aiken et al., 1984; Harris and Sandifer, 1986; Akarasanon et al., 2004; Feng et

al., 2019). Further investigation is needed to determine the optimal time interval for regenerating spermatophores between repeated electroejaculation episodes. Additionally, the effects of numerous and repeated attempts on the success rate of spermatophore collection and sperm quality should be examined, along with approaches to improve the success rate.

4.6. Conclusions

The probability of success when collecting spermatophores from wild-caught redclaw crayfish weighing between 60 and 130 g using electroejaculation was 15 – 30 %. In addition, the use of conventional and advanced sperm quality species-specific diagnostic tools was helpful in studying the effects of different spermatophore collection methods on male reproductive potential in redclaw crayfish. Spermatozoa recovered from spermatophores removed by dissection were of greater quality than those recovered by electroejaculation. This suggests that it may be preferential to use sperm recovered by dissection rather than electroejaculation for use in selective breeding programs utilising cryopreservation and artificial fertilisation in the future, although male broodstock will need to be sacrificed. Conversely, electroejaculation allows for the collection of spermatophores and the evaluation of sperm quality from redclaw male broodstock intended for future breeding and production.

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4.8. References

Aiken, D.E., Waddy, S.L., Moreland, K., Polar, S.M., 1984. Electrically induced ejaculation and artificial insemination of the american lobster *Homarus Americanus*. J. Crust. Biol. 4(4), 519-527. https://doi.org/https://doi.org/10.2307/1548065.

Akarasanon, K., Damrongphol, P., Poolsanguan, W., 2004. Long-term cryopreservation of spermatophore of the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). Aquac. Res. 35(15), 1415-1420. https://doi.org/https://doi.org/10.1111/j.1365-2109.2004.01163.x.

Anand, P., C. P, B., Aravind, R., Francis, B., Rajan, R., T N, V., Panigrahi, A., Sudheer, N.S., Rajamanickam, S., Kumar, S., Antony, J., Kondusamy, A., Karingalakkandy Poochirian, J., Kk, V., 2023. Reproductive performance of captive-reared Indian white shrimp, *Penaeus indicus*, broodstocks over two generations. Front. Mar. Sci. 10, 1101806. https://doi.org/10.3389/fmars.2023.1101806.

Anel-López, L., García-Álvarez, O., Parrilla, I., Del Olmo, D., Fernández-Santos, M.R., Soler, A.J., Maroto-Morales, A., Ortiz, J.A., Alkmin, D.V., Tarantini, T., Roca, J., Martínez, E.A., Vazquez, J.M., Garde, J.J., 2016. The effect of oxidative stress on Thawed Bulk-Sorted Red Deer sperm. Reprod. Domest. Anim. 51(3), 407-414. https://doi.org/10.1111/rda.12694.

Aquino, J.I.L., Elliott, L., Zeng, C., Paris, D.B.B.P., 2022. Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation, and their potential application to decapod crustacean aquaculture. Rev. Aquac. 14(2), 848-889. https://doi.org/https://doi.org/10.1111/raq.12627.

Bambozzi, A.F., Mattos, L., Mello, M., Oshiro, L., 2014. Post-mortem spermatophore and sperm cryopreservation of the white shrimp *Litopenaeus schmitti*. Bol. Inst. Pesca 40, 49-60.

Bart, A.N., Choosuk, S., Thakur, D.P., 2006. Spermatophore cryopreservation and artificial insemination of black tiger shrimp, *Penaeus monodon* (Fabricius). Aquac. Res. 37(5), 523-528. https://doi.org/https://doi.org/10.1111/j.1365-2109.2006.01460.x.

Beach, D., Talbot, P., 1987. Ultrastructural comparison of sperm from the crayfishes *Cherax tenuimanus* and *Cherax albidus*. J. Crust. Biol. 7(2), 205-218. https://doi.org/https://doi.org/10.2307/1548602.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J.K., Peixoto, S., Robeck, T.R., Cabrita, E., 2019. Sperm handling in aquatic animals for artificial reproduction. Theriogenology 133, : 161-178. https://doi.org/https://doi.org/10.1016/j.theriogenology.2019.05.004.

Bitomsky, J., 2008. Scoping report- Redclaw industry development, in: Lofthouse, L. (Ed.). 36 Grafton Street, Cairns, QLD, 4870, pp. 22-40.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165(3), 535-548. https://doi.org/https://doi.org/10.1016/j.ygcen.2009.02.011.

Braga, A.L., Nakayama, C.L., Martins, J.G., Colares, E.P., Wasielesky, W., 2010. Spermatophore quality of the pink shrimp *Farfantepenaeus paulensis* (Decapoda, Dendrobranchiata) broodstock fed with different maturation diets. Aquaculture 307(1), 44-48. https://doi.org/https://doi.org/10.1016/j.aquaculture.2010.07.010.

Bugnot, A.B., López Greco, L.S., 2009. Sperm production in the red claw crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). Aquaculture 295(3), 292-299. https://doi.org/https://doi.org/10.1016/j.aquaculture.2009.07.021.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V., 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432, 389-401. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.04.034.

Cabrita, E., Robles, V., Herráez, M.P., 2009. Sperm quality assessment, in: Cabrita, E., Robles, V., Herráez, M.P. (Eds.), Methods in Reproductive Aquaculture: Marine andFreshwater Species. Biology Series. CRC Press (Taylor and Francis Group), Boca Raton, Florida, USA, pp. 93-148.

Carmona-Osalde, C., Rodriguez-Serna, M., Olvera-Novoa, M.A., Gutierrez-Yurrita, P.J., 2004. Gonadal development, spawning, growth and survival of the crayfish *Procambarus llamasi* at three different water temperatures. Aquaculture 232(1), 305-316. https://doi.org/https://doi.org/10.1016/S0044-8486(03)00527-1.

Castelo-Branco, T., Batista, A.M., Guerra, M.M.P., Soares, R., Peixoto, S., 2015. Sperm vitrification in the white shrimp *Litopenaeus vannamei*. Aquaculture 436, : 110-113. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.11.005.

Castelo-Branco, T., Quinto, B.P.T., Soares, R., Guerra, M.M.P., Peixoto, S., 2016. Cadaveric sperm viability in the white shrimp *Litopenaeus vannamei*. Aquac. Res. 47(10), 3350-3351. https://doi.org/https://doi.org/10.1111/are.12770.

Chamberlain, G., Lawrence, A.L., 1981. Maturation, reproduction, and growth of *Penaeus vannamei* and *P. stylirostris* fed natural diets. J. World Maric. Soc. 12, 207-224.

Devaux, A., Fiat, L., Gillet, C., Bony, S., 2011. Reproduction impairment following paternal genotoxin exposure in brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*). Aquat. Toxicol. 101(2), 405-411. https://doi.org/https://doi.org/10.1016/j.aquatox.2010.11.017.

Diggles, B.K., 2019. Review of some scientific issues related to crustacean welfare. ICES J. Mar. Sci 76(1), 66-81. https://doi.org/https://doi.org/10.1093/icesjms/fsy058.

Duangjai, E., Umyoo, P., Meunpol, O., Puanglarp, N., 2023. Sperm quality assessment of white shrimp (*Litopenaeus vannamei*) broodstock using comet assay. Trends Sci. 20(6), 4768. https://doi.org/10.48048/tis.2023.4768.

FAO-FIGIS, 2019. Global fisheries and aquaculture production 1950 - 2017, Fisheries Global Information System (FIGIS). FI Institutional Websites. Statistics and Information Branch. Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153, Rome.

Farhadi, A., Harlıoğlu, M.M., Gür, S., 2019a. Artificial extrusion of spermatophores for insemination of the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Aquac. Rep. 14(100200), 1-5. https://doi.org/https://doi.org/10.1016/j.aqrep.2019.100200.

Farhadi, A., Harlıoğlu, M.M., Harlıoğlu, A.G., 2019b. The relationship between weight and length of male broodstock with gamete production in the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Aquac. Res. 50(4), 1380-1383. https://doi.org/https://doi.org/10.1111/are.13982.

Feng, T., Gosálvez, J., Lopez-Fernandez, C., Arroyo, F., Paterson, B., Johnston, S., 2018. Sperm chromatin dispersion test (SCDt) for the assessment of sperm DNA fragmentation in black tiger prawn, *Penaeus monodon*. Aquaculture 491, : 281-288. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.03.041.

Feng, T., Paterson, B., Coman, G., Johnston, S., 2019. Evaluating the impact of moulting and chilled storage of spermatophores on the integrity of plasma membrane, acrosome and DNA of black tiger prawn (*Penaeus monodon*) spermatozoa. Aquac. Res. 50(1), 226-235. https://doi.org/https://doi.org/10.1111/are.13888.

Fidan, A.F., Yeni, D., Avdatek, F., ÖZÇInar, Ü., Hazman, Ö., 2018. Changes in oxidative stress markers during electro-ejaculation procedure in merino rams. J. Hellenic Vet. Med. Soc. 68(3), 479-486. https://doi.org/10.12681/jhvms.15544.

Galeotti, P., Bernini, G., Locatello, L., Sacchi, R., Fasola, M., Rubolini, D., 2012. Sperm traits negatively covary with size and asymmetry of a secondary sexual trait in a freshwater crayfish. *PLoS One* 7(8), e43771-e43771. https://doi.org/https://doi.org/10.1371/journal.pone.0043771.

Ghanawi, J., Saoud, I.P., 2012. Molting, reproductive biology, and hatchery management of redclaw crayfish *Cherax quadricarinatus* (von Martens 1868). Aquaculture 358-359, 183-195. https://doi.org/https://doi.org/10.1016/j.aquaculture.2012.06.019.

Goñi, R., Quetglas, A., Reñones, O., 2003. Size at maturity, fecundity and reproductive potential of a protected population of the spiny lobster *Palinurus elephas* (Fabricius, 1787) from the western Mediterranean. Mar. Biol. 143(3), 583-592. https://doi.org/10.1007/s00227-003-1097-5.

González-Marín, C., Gosálvez, J., Roy, R., 2012. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. Int. J. Mol. Sci. 13(11), 14026-14052. https://doi.org/10.3390/ijms131114026.

Grimard, B., de Boyer des Roches, A., Coignard, M., Lehébel, A., Chuiton, A., Mounier, L., Veissier, I., Guatteo, R., Bareille, N., 2019. Relationships between welfare and reproductive performance in French dairy herds. Vet. J. 248, 1-7. https://doi.org/https://doi.org/10.1016/j.tvjl.2019.03.006.

Harris, S.E.G., Sandifer, P.A., 1986. Sperm production and the effects of electrically induced spermatophore expulsion in the prawn *Macrobrachium Rosenbergii* (De Man). J. Crust. Biol. 6(4), 633-647. https://doi.org/https://doi.org/10.1163/193724086X00433.

Haubrock, P.J., Oficialdegui, F.J., Zeng, Y., Patoka, J., Yeo, D.C.J., Kouba, A., 2021. The redclaw crayfish: A prominent aquaculture species with invasive potential in tropical and subtropical biodiversity hotspots. Rev. Aquac. 13(3), 1488-1530. https://doi.org/https://doi.org/10.1111/raq.12531.

Iommiello, V.M., Albani, E., Di Rosa, A., Marras, A., Menduni, F., Morreale, G., Levi, S.L., Pisano, B., Levi-Setti, P.E., 2015. Ejaculate oxidative stress is related with sperm DNA fragmentation and round cells. Int. J. Endocrinol. 2015, 321901. https://doi.org/10.1155/2015/321901.

Irvin, S., Coman, G., Musson, D., Doshi, A., Stokes, C., 2018. Aquaculture viability. A technical report to the Australian Government from the CSIRO Northern Australia Water Resource Assessment, part of the National Water Infrastructure Development Fund: Water Resource Assessments, CSIRO, Australia.

Jerry, D.R., 2001. Electrical stimulation of spermatophore extrusion in the freshwater yabby (*Cherax destructor*). Aquaculture 200(3), 317-322. https://doi.org/https://doi.org/10.1016/S0044-8486(01)00511-7.

Jiménez-Rabadán, P., Ramón, M., García-Álvarez, O., Maroto-Morales, A., del Olmo, E., Pérez-Guzmán, M.D., Bisbal, A., Fernández-Santos, M.R., Garde, J.J., Soler, A.J., 2012. Effect of semen collection method (artificial vagina vs. electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates. Anim. Reprod. Sci. 132(1-2), 88-95. https://doi.org/10.1016/j.anireprosci.2012.04.005.

Jones, C., 2011. Cultured aquatic species information programme- *Cherax quadricarinatus*, Cultured Aquatic Species Information Programme, 13 January 2011 ed. FAO Fisheries and Aquaculture Department, Rome.

Jones, C.M., 1995. Production of juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae) I. Development of hatchery and nursery procedures. Aquaculture 138(1), 221-238. https://doi.org/https://doi.org/10.1016/0044-8486(95)00068-2.

Jones, C.M., Ruscoe, I.M., Queensland, F.F.a.A.C., 1996. Production technology for redclaw crayfish (*Cherax quadricarinatus*): Final report. Freshwater Fisheries and Aquaculture Centre, Department of Primary Industries, Walkamin, QLD.

Jones, C.M., Valverde, C., 2020. Development of mass production hatchery technology for the redclaw crayfish, *Cherax quadricarinatus*. Freshw. Crayfish 25(1), 1 - 6. https://doi.org/10.5869/fc.2020.v25-1.001.

King, C., 1993. Potential fecundity of redclaw crayfish, *Cherax quadricarinatus* von Martens, in culture. Aquaculture 114(3), 237-241. https://doi.org/https://doi.org/10.1016/0044-8486(93)90299-E.

Koteeswaran, R., Pandian, T.J., 2002. Live sperm from post-mortem preserved Indian catfish. Current Science 82(4), 447-450.

Kouba, A., Niksirat, H., Bláha, M., 2015. Comparative ultrastructure of spermatozoa of the redclaw *Cherax quadricarinatus* and the yabby *Cherax destructor* (Decapoda, Parastacidae). Micron 69, : 56-61. https://doi.org/https://doi.org/10.1016/j.micron.2014.11.002.

Kowalski, R.K., Cejko, B.I., 2019. Sperm quality in fish: Determinants and affecting factors. Theriogenology 135, 94-108. https://doi.org/https://doi.org/10.1016/j.theriogenology.2019.06.009.

Kubec, J., Niksirat, H., Kouba, A., Kozák, P., 2012. The electrostimulation of spermiation in signal crayfish (*Pacifastacus Leniusculus*). Bulletin VÚRH Vodňany 48, 5-15.

Leung-Trujillo, J.R., Lawrence, A.L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65(3), 363-370. https://doi.org/https://doi.org/10.1016/0044-8486(87)90249-3.

Li, M., Dong, W.-j., Xiu, J.-y., Wang, Y.-f., 2007. The effect of vitamin E in diet on spawning of red swamp crayfish *Procambarus clarkii* Girard. J. Fish. China 2007-S1.

Liu, S., Gong, S., Li, J., Huang, W., 2013. Effects of water temperature, photoperiod, eyestalk ablation, and non-hormonal treatments on spawning of ovary-mature red swamp crayfish. N. Am. J. Aquac. 75(2), 228-234. https://doi.org/10.1080/15222055.2012.746247.

Masser, M.P., Rouse, D.B., 1997. Australian red claw crayfish, Southern Regional Aquaculture Center Publication. United States Department of Agriculture, Cooperative States Research, Education, and Extension Service, United States of America, pp. 1-7.

Medley, P.B.C., Jones, C., W. Jr Avault, J., 1994. A global perspective of the culture of Australian redclaw crayfish, *Cherax quadricarinatus*: Production, economics and marketing. J. World Aquac. Soc. 25(4), 6-13.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Rev. Aquac. 5(s1), S194-S223. https://doi.org/https://doi.org/10.1111/raq.12025.

Mobsby, D., 2018. Australian fisheries and aquaculture statistics 2017, Fisheries Research and Development Corporation Project 2018-134. ABARES, Canberra, December. CC BY 4.0, pp. 1-94.

Nakayama, C., Peixoto, S., Lopes, D., Vita, G., Krummenauer, D., Foes, G., Cavalli, R., Wasielesky, W., 2008. Manual and electric extrusion methods of spermatophores of wild breeders of *Farfantepenaeus paulensis* (Decapoda: Penaeidae). Cienc. Rural. 38(7), 2018-2022. https://doi.org/https://doi.org/10.1590/S0103-84782008000700034.

Niksirat, H., Andersson, L., James, P., Kouba, A., Kozák, P., 2014a. Proteomic profiling of the signal crayfish *Pacifastacus leniusculus* egg and spermatophore. Anim. Reprod. Sci. 149(3), 335-344. https://doi.org/https://doi.org/10.1016/j.anireprosci.2014.07.024.

Niksirat, H., James, P., Andersson, L., Kouba, A., Kozák, P., 2015. Label-free protein quantification in freshly ejaculated versus post-mating spermatophores of the noble crayfish *Astacus astacus*. J. Proteom. 123, : 70-77. https://doi.org/https://doi.org/10.1016/j.jprot.2015.04.004.

Niksirat, H., Kouba, A., Kozák, P., 2014b. Post-mating morphological changes in the spermatozoon and spermatophore wall of the crayfish *Astacus leptodactylus*: Insight into a non-motile spermatozoon. Anim. Reprod. Sci. 149(3), 325-334. https://doi.org/https://doi.org/10.1016/j.anireprosci.2014.07.017.

Niksirat, H., Vancová, M., Andersson, L., James, P., Kouba, A., Kozák, P., 2016. Protein modification in the post-mating spermatophore of the signal crayfish *Pacifastacus*

leniusculus: insight into the tyrosine phosphorylation in a non-motile spermatozoon. Anim. Reprod. Sci. 172, : 123-130. https://doi.org/https://doi.org/10.1016/j.anireprosci.2016.07.009.

Parnes, S., Sagi, A., 2002. Intensification of redclaw crayfish *Cherax quadricarinatus* culture. I. Hatchery and nursery system. Aquac. Eng. 26(4), 251-262. https://doi.org/https://doi.org/10.1016/S0144-8609(02)00034-1.

Peña-Almaraz, O.A., Cortés-Jacinto, E., Vega-Villasante, F., Arcos-Ortega, G.F., Badillo-Zapata, D., Vargas-Ceballos, M.A., 2022. Sperm viability in wild-caught males of *Macrobrachium tenellum* (Smith, 1871)(Decapoda: Caridea: Palaemonidae) fed with different diets. Nauplius 30, 1 - 14.

Pérez-Cerezales, S., Martínez-Páramo, S., Beirão, J., Herráez, M.P., 2010. Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 139(6), 989. https://doi.org/10.1530/rep-10-0037.

Pérez-Rodríguez, J.C., Gómez-Gutiérrez, J., López-Greco, L.S., Cortés-Jacinto, E., 2019. Spermatophore production and sperm quality of the river prawn *Macrobrachium americanum* Spence Bate, 1868 fed with different diets. Aquac. Res. 0(0), 1-13. https://doi.org/https://doi.org/10.1111/are.14265.

Pratoomchaat, B., Piyatiratitivorakul, S., Menasveta, P., Fast, A.W., 1993. Sperm quality of pond-reared and wild-caught *Penaeus monodon* in Thailand. J. World Aquac. Soc. 24(4), 530-540. https://doi.org/https://doi.org/10.1111/j.1749-7345.1993.tb00584.x.

R.Core.Team, 2022. R: A language and environment for statistical computing, R Foundation for Statistical Computing. Vienna, Austria.

Rendón Rodríguez, S., Macías Regalado, E., Calderón Pérez, J.A., Núñez Pastén, A., Solís Ibarra, R., 2007. Comparison of some reproductive characteristics of farmed and wild white shrimp males *Litopenaeus vannamei* (Decapoda: Penaeidae). Rev. Biol. Trop. 55(1), 199-206. https://doi.org/10.15517/rbt.v55i1.6071.

Restelli, A.E., Bertolla, R.P., Spaine, D.M., Miotto, A., Jr., Borrelli, M., Jr., Cedenho, A.P., 2009. Quality and functional aspects of sperm retrieved through assisted ejaculation in men with spinal cord injury. Fertil. Steril. 91(3), 819-825. https://doi.org/10.1016/j.fertnstert.2007.12.060.

Riesco, M.F., Valcarce, D.G., Martínez-Vázquez, J.M., Robles, V., 2019. Effect of low sperm quality on progeny: a study on zebrafish as model species. Sci. Rep. 9(1), 11192. https://doi.org/10.1038/s41598-019-47702-7.

Rigg, D., Seymour, J., Courtney, R., Jones, C., 2020. A review of juvenile redclaw crayfish *Cherax quadricarinatus* (von Martens, 1898) production in aquaculture. Freshw. Crayfish 25(1), 13-30. https://doi.org/10.5869/fc.2020.v25-1.013.

Rodriguez-Gonzalez, H., Hernandez-Llamas, A., Garcia-Ulloa, M., Villarreal, H., 2006. Effect of dietary protein level on spawning and egg quality of redclaw crayfish *Cherax quadricarinatus*. Aquaculture 257(1/4), 412-419.

Rodríguez-González, H., Villarreal, H., García-Ulloa, M., Hernández-Llamas, A., 2009. Dietary lipid requirements for optimal egg quality of redclaw crayfish, *Cherax quadricarinatus*. J. World Aquac. Soc. 40(4), 531-539. https://doi.org/10.1111/j.1749-7345.2009.00267.x.

Rodríguez-González, H., Villarreal, H., Hernández-Llamas, A., García-Ulloa, M., Vázquez-Boucard, C., Serrano-Pinto, V., 2011. Effect of dietary protein-to-energy ratio on reproduction in female redclaw crayfish *Cherax quadricarinatus*. Aquaculture Research 42(12), 1840-1847. https://doi.org/10.1111/j.1365-2109.2010.02787.x.

Rubolini, D., Galeotti, P., Pupin, F., Sacchi, R., Nardi, P.A., Fasola, M., 2007. Repeated matings and sperm depletion in the freshwater crayfish *Austropotamobius italicus*. Freshw. Biol. 52(10), 1898-1906. https://doi.org/10.1111/j.1365-2427.2007.01814.x.

Sainte-Marie, B., 2007. Sperm demand and allocation in decapod crustaceans, in: Duffy, J.E., Thiel, M. (Eds.), Evolutionary ecology of social and sexual systems: Crustaceans as model organisms. Oxford University Press, p. 0.

Samuel, M.J., Kannupandi, T., Soundarapandian, P., 1999. Nutritional effects on male reproductive performance in the freshwater prawn *Macrobrachium malcolmsonii* (H. Milne Edwards). Aquaculture 172(3), 327-333. https://doi.org/https://doi.org/10.1016/S0044-8486(98)00500-6.

Sandifer, P.A., Lawrence, A.L., Harris, S.G., Chamberlain, G.W., Stokes, A.D., Bray, W.A., 1984. Electrical stimulation of spermatophore expulsion in marine shrimp, *Penaeus* spp. Aquaculture 41(2), 181-187. https://doi.org/https://doi.org/10.1016/0044-8486(84)90096-6.

Sato, T., Yoseda, K., Abe, O., Shibuno, T., 2008. Male maturity, number of sperm, and spermatophore size relationships in the coconut crab *Birgus Latro* on Hatoma Island, Southern Japan. J. Crust. Biol. 28(4), 663-668. https://doi.org/https://doi.org/10.1651/07-2966.1.

Selvakumar, N., Krishnamoorthy, D., Beulah, C.D., Natesan, M., 2018. Retrieval and cryopreservation of sperm in spermatophores from cadaveric Indian white shrimp, *Fenneropenaeus indicus* (H. Milne Edwards, 1837). Anim. Reprod. Sci 192, 185-192. https://doi.org/https://doi.org/10.1016/j.anireprosci.2018.03.008.

Yeh, H.-S., Rouse, D.B., 1994. Indoor spawning and egg development of the red claw crayfish *Cherax quadricarinatus*. J. World Aquac. Soc. 25(2), 297-302. https://doi.org/10.1111/j.1749-7345.1994.tb00194.x.

Chapter 5. Reproductive potential of male redclaw crayfish (*Cherax quadricarinatus*) fed with plant-based diets supplemented with astaxanthin and/or cholesterol

5.1. Abstract

Redclaw crayfish exhibit desirable characteristics that render them suitable for commercial aquaculture. However, rudimentary and inconsistent hatchery technology restricts redclaw industry expansion. Among management strategies in the hatchery, nutritional input has a crucial impact on gonadal maturation and spawning success in farmed crustaceans. In particular, dietary astaxanthin and cholesterol have proven beneficial to gonadal maturation and sperm quality in other crustaceans. This study evaluates, for the first time, the effects of astaxanthin (AX) and cholesterol (CHO) supplemented diets, on sperm quality and other reproductive performance indicators in redclaw male broodstock, over a 60-day feeding trial. Four diets were formulated, including a base control (no supplement) diet and base diets supplemented with 100 mg/kg AX, 10 g/kg CHO, or both 100 mg/kg AX and 10 g/kg CHO. Diets were fed to six replicate groups of wild-caught mature redclaw males (n = 48 per treatment) twice daily for 60 days. Reproductive performance indicators, including sperm viability, concentration, spermatophore weight, gonadosomatic (GSI), and hepatosomatic indices (HSI), increased over the duration of the experiment ($p < 0.05$). In contrast, sperm DNA fragmentation doubled over the same period ($p < 0.001$). Overall, implementation of a plant-based broodstock diet supplemented with AX or CHO accelerated gonadal maturation within 30 days, indicating earlier attainment of reproductive capacity, albeit with decreased sperm DNA integrity. Notably, the broodstock basal diet formulation in this study may prove

beneficial for future breeding technologies to accelerate gonadal development and enhance gamete quality in intensive hatchery systems.

Keywords: Broodstock nutrition, DNA fragmentation, flow cytometry, redclaw, sperm quality, TUNEL assay

5.2. Introduction

Redclaw crayfish (*Cherax quadricarinatus*) have the potential to be a valuable freshwater crustacean for commercial aquaculture (Jones et al., 1996; Rigg et al., 2020). For more than four decades, the production of redclaw juveniles for grow-out operations has been modified from traditionally extensive and semi-intensive ponds (Medley et al., 1994; Jones et al., 1996) to more intensive systems, including indoor and controlled hatchery juvenile production (Jones, 1995a, 1995b; Parnes and Sagi, 2002; Jones and Valverde, 2020; Rigg et al., 2020). Controlled hatchery conditions facilitate environmental and nutritional interventions to enhance hatchery production and optimise gamete quality. For farmed species such as redclaw, the quality of offspring relies primarily on the gamete quality of the broodstock (Migaud et al., 2013). The aim of broodstock conditioning and spawning is to optimise both the quality and quantity of offspring. Appropriate nutrition is important during broodstock conditioning to supply the necessary nutrients and energy for the onset of gonadal maturation and subsequent embryonic development (Saoud et al., 2013). In other commercially important crustaceans, such as shrimps, the nutritional value of broodstock diets has been shown to affect sperm quantity and quality significantly (Samuel et al., 1999; Harlioğlu et al., 2013; Leelatanawit et al., 2014; Pérez-Rodríguez et al., 2019). Broodstock nutrition is, therefore, a key modifiable factor that could be targeted to improve the

reproductive capacity of breeders and larval production in commercial redclaw hatcheries (Bray and Lawrence, 1998; Izquierdo et al., 2001).

Efforts to intensify redclaw crayfish production have included investigation of species-specific dietary nutritional requirements and formulations; however, studies have primarily focused on juveniles (Cortés-Jacinto et al., 2003; Thompson et al., 2003; Cortés-Jacinto et al., 2004; Cortés-Jacinto et al., 2005; Thompson et al., 2005; Zenteno-Savín et al., 2008; Stumpf et al., 2010; Stumpf et al., 2014; Stumpf et al., 2019; Rigg et al., 2020; Chen et al., 2021; López-Greco et al., 2022), pre-adults (Cortés-Jacinto et al., 2004; Pavasovic et al., 2007b; Méndez-Martínez et al., 2021; Shehata et al., 2023), and adults (Pavasovic et al., 2006; Pavasovic et al., 2007a). Some studies have explored dietary composition and supplementation effects on female redclaw broodstock, focusing mainly on ovarian maturation (Lu et al., 2020; López-Greco et al., 2022). Although diets formulated for enhancing redclaw juvenile growth covered some somatic growth requirements, the formulation of diets for gonadal maturation and sperm quality, especially for male broodstock, requires further study. In addition, the potential dietary effects on male fertility remain poorly investigated.

During reproductive maturation, a high energy-dependent process, crustacean's physiological responses change in accordance with nutritional inputs, prioritising gonadal development over pure somatic growth (Harrison, 1990; Volkoff and London, 2018). Changes include a drop in antioxidant levels during gametogenesis and spermiation, increased formation of reactive oxygen species (ROS; Félix et al., 2021) and an upward shift in steroid hormone production for moulting and reproduction (Kumar et al., 2018). Notably, increased oxidative stress and release of ROS during spermatogenesis are the leading cause of DNA damage in

spermatozoa (Wagner et al., 2018). Astaxanthin (AX), a carotenoid pigment, prevents peroxidation or oxidative damage in reproductive cells, tissues, and embryos by quenching excessive amounts of ROS and free radicals (Miki, 1991; Lim et al., 2018), thereby positively influencing crustaceans' gonadal maturation and reproduction (Meyers and Latscha, 1997; Lim et al., 2018). Cholesterol (CHO) is considered essential for crustaceans but is in low abundance in redclaw feeds that are largely plant-based. Cholesterol is a precursor of steroid hormones that regulate moulting and reproduction in crustaceans and plays an important role in vitellogenesis (Teshima, 1997; Kumar et al., 2018). Both AX and CHO are essential nutrients that cannot be synthesised *de novo* in crustaceans and are obtained from exogenous dietary sources (Meyers and Latscha, 1997; Teshima, 1997). While AX and CHO supplementation has proven beneficial to gonadal maturation in female crustaceans (Pangantihon-Kühlmann et al., 1998; Liñán-Cabello et al., 2004; Hou et al., 2022), their effects on the reproductive performance of male freshwater crayfish have not been investigated.

The role of sperm quality in crustacean hatcheries is often overlooked. While female egg development is energetically expensive, spermatozoa are usually viewed as a simple DNA delivery mechanism. However, recent studies have shown that poor sperm quality, primarily due to DNA damage, can significantly impede fertilisation and lead to adverse reproductive outcomes (Pérez-Cerezales et al., 2010; Simon et al., 2014; Riesco et al., 2019; Duangjai et al., 2023). Spermatozoa quality can be retrospectively determined by their capacity to produce viable embryos when in contact with good-quality eggs under favourable environmental conditions (Bobe and Labbé, 2010). Other aquaculture species use fertilisation rates to represent male reproductive potential, but this technique has not been established for redclaw crayfish. While estimating fertility rates is effective, a major disadvantage is that

data is retrospectively captured, which delays the screening of males for breeding programs. In addition, several variables could influence the results, including egg quality, spermatozoa/egg ratio, incubation time and other environmental and nutritional conditions, making standardised assessment difficult (Cabrita et al., 2009). As a result, commercial aquaculture has considered evaluating sperm quality to diagnose early potential fertility of male breeders for selective breeding programs, reducing operational costs (Cabrita et al., 2009; Feng, 2018).

Recently, efforts have been made to improve the sensitivity of diagnostic tools using nuclear staining and flow cytometry to estimate potential fertilising capacity in male decapod crustaceans (Feng et al., 2018; Feng et al., 2019; Aquino et al., 2022; Duangjai et al., 2023). In redclaw males, evaluation of sperm quality, including the assessment of sperm concentration, viability and DNA integrity, was developed and optimised (Aquino unpublished - Chapter 3). This study used conventional and advanced sperm diagnostic tools to investigate the effects of a plant-based diet supplemented with AX, CHO, or both nutrients on sperm quality and other reproductive indicators in redclaw crayfish.

5.3. Material and Methods

5.3.1. Animals

Male redclaw crayfish were collected using baited funnel traps (Jones, 1990) during winter in July 2022 from a dry tropical region of northern Queensland (-19°24'44.28" S, 146°48'36" E). Baited traps were set adjacent to riverbanks no deeper than two meters late in the afternoon and were collected the following morning. Sexually mature redclaw males (88.1 ± 1.1 g)

were transported to the Marine and Aquaculture Research Facility (MARFU), James Cook University, recirculating facility for disinfection. Disinfection involved immersion in salt water (30 ppt) for 20 min before placement in a clean tank with vigorous aeration (Sugiani et al., 2015). An exemption was granted by the James Cook University Animal Ethics Committee as ethics approval was not required for crustacean studies. This study conforms to Marine & Aquaculture Research Facility, James Cook University procedures for ethical animal treatment and best practices.

5.3.2. Feeding regime

5.3.2.a. Experimental design and diet preparation

Four experimental dietary treatments were formulated to evaluate the feeding of a plant-based diet supplemented with AX, CHO, or both ingredients (AX + CHO) on the reproductive performance of male redclaw broodstock over a period of 60 days (Figure 5.1). The basal diet, without the inclusion of AX or CHO, served as the control dietary treatment (Table 5.1). The diet was formulated to contain 38.5 % protein, 9.5 % lipid and a gross energy of 5,072 kcal/100 g. Dry ingredients, excluding premixes and additives, were ground using a Rotor Beater Mill (SR-300, Retsch, Haan, Germany) with a 750 µm screen and weighed to the nearest 0.01 g before mixing (Hobart A200 N Planetary Mixer, Troy, Ohio, USA) along with feed additives (antioxidants, vitamins, and minerals, astaxanthin, and/or cholesterol). Oil and sufficient water were added to facilitate pelleting (approximately 30 %). Pellets were then formed through a 3 mm die (Hobart A120 Planetary Mixer with mincer attachment, Hobart, Australia), steamed for 5 min, then dried (TD 700-F Premium Dryer Thermoline, Wetherill Park, NSW) for 6 h to attain a residual moisture content of 10 %. Dried pellets were stored in

air-tight containers covered with aluminium foil to prevent light exposure and kept at - 20 °C until use.

Table 5.1. Composition of the experimental diet.

Feed ingredients (g kg^{-1})	Control	Astaxanthin (AX)	Cholesterol (CHO)	AX + CHO
Defatted soybean meal	300.0	300.0	300.0	300.0
Wheat gluten	200.0	200.0	200.0	200.0
Lupin meal	200.0	200.0	200.0	200.0
Canola oil	40.0	40.0	40.0	40.0
Flaxseed oil	40.0	40.0	40.0	40.0
Vitamin E	0.1	0.1	0.1	0.1
Monocalcium phosphate	14.0	14.0	14.0	14.0
Antioxidant [(a)]	1.0	1.0	1.0	1.0
Vitamin mixture [(b)]	5.0	5.0	5.0	5.0
Mineral mixture [(c)]	1.8	1.8	1.8	1.8
Whole wheat flour	198.1	197.1	188.1	187.1
Cholesterol [(d)]	-	-	10.0	10.0
Astaxanthin 10 % [(e)]	-	1.0	-	1.0
Total	1000	1000	1000	1000

[a] Santoquin (66 % ethoxyquin).

[b] Vitamin premix (g kg^{-1}): A (3,000 IU), D_3 (24 IU), K (10.0), inositol (250.0), nicotinic acid B_3 (45.0), pantothenic acid B_5 (10.0), folic acid (5.0), riboflavin B_2 (20.0), cyanocobalamin

B_{12} (0.05), biotin (1.0), pyridoxine B_6 (10.0), thiamine B_1 (10.0), vitamin C (150.0), and antioxidant (15.0) and dextrose was used a carrier.

[c] Mineral premix (g kg^{-1}): Cu (6.0), Co (12.5), Mn (19.0), I (1.0), Se (0.5), Fe (125.0), and Zn (66.0).

[d] VWR Lifescience, Ohio, USA

[e] Carophyll Pink, DSM, Heerlen, Netherlands

Each dietary treatment was randomly assigned to six replicates of a circular 100-L tank. Each tank housed eight males (n = 48/ treatment) and eight polyvinyl chloride hides 6 cm in diameter x 18 cm in length. Water temperature was maintained at 26.8 ± 0.1 °C, pH at 8.1 ± 0.04, and dissolved oxygen at 6.70 ± 0.01 ppm, with a single recirculating system which maintained total ammonia nitrogen (TAN; NH_4^+-N/ NH_3-N), nitrite-N (NO_2^--N), and nitrate-N (NO_3^--N) at 0.1 ± 0.0, 0.0 ± 0.0, and 31.5 ± 3.7 ppm, respectively. The freshwater recirculating aquaculture system employs mechanical, biological and UV filtration systems. It has a holding capacity of 6 tons of dechlorinated freshwater with an exchange flow rate of 250 L/min. Photoperiod was controlled at 14 h L: 10 h D.

5.3.2.b. Crayfish maintenance and sampling

All crayfish were fed the basal diet during an acclimation period of 15 days (Days -15 to 0). After acclimation, crayfish were fed their respective supplemented diets twice daily at 1.5 % of their body weight at about 10:00 and 17:00 h, with 40 % being fed in the morning and 60 % being fed in the afternoon from 0 to 60 days. Mortality and moulting were monitored and recorded twice daily.

Sampling for assessment of sperm quality was conducted on Days 0, 30 and 60 (Figure 5.1). Crayfish at the intermoult stage were anaesthetised in chilled freshwater (15 °C) for 10 min, body moisture was removed using a paper towel, and then weighed. After weighing, surface bacteria on the crayfish were removed by swabbing with 80 % ethanol. Gonads, hepatopancreas, and spermatophore were collected and weighed.

5.3.2.c. Spermatophore extraction and sperm preparation

The dissection protocol described in Chapter 4 was used to collect spermatophores from male redclaw. The suspension of spermatozoa was prepared following the Chapter 3 protocol.

5.3.3. Sperm quality assessment

5.3.3.a. Sperm concentration

The protocol for estimating sperm concentration in each spermatophore was described in Chapter 3.

5.3.3.b. Sperm viability

The procedure described in Chapter 3 was followed to evaluate sperm viability in redclaw. Briefly, Hoechst 33342 (H_{342}; Sigma-Aldrich Pty. Ltd., Macquarie Park NSW, Australia) and propidium iodide (PI; Sigma-Aldrich Pty. Ltd., Macquarie Park NSW, Australia) nuclear staining followed by flow cytometry analysis were used to determine the integrity of spermatozoa plasma membrane (expressed in percentage).

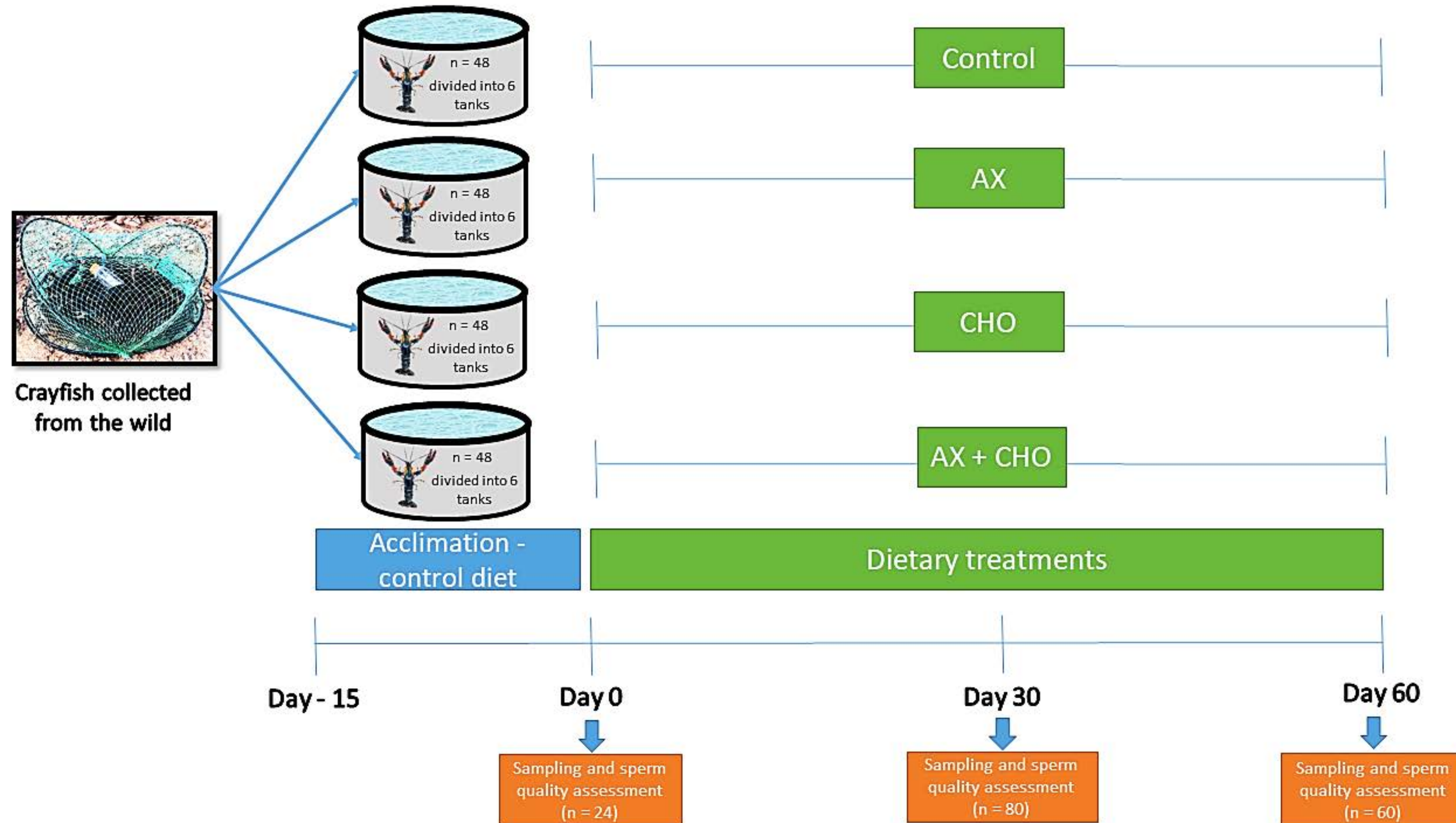

Figure 5.1. The schematic diagram of the experimental design aimed to evaluate supplementation with a plant-based diet with either astaxanthin, cholesterol, or both ingredients on the reproductive potential of male redclaw broodstock during a supplementation period of 60 days. AX - astaxanthin; CHO - cholesterol.

5.3.3.c. DNA fragmentation assay

The degree of DNA fragmentation was assessed using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method with slight modifications from the manufacturer's instructions (*In Situ* Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Mannheim, Germany), following the protocol described in Chapter 3. Flow cytometry was used to quantify DNA-damaged spermatozoa (expressed in percentage).

5.3.3.d. Flow cytometry

The methods described in Chapter 3 were used to perform the flow cytometry analysis.

5.3.3.e. Calculation of total potential fertile sperm concentration (TPFSC), gonadosomatic (GSI) and hepatosomatic (HSI) indices

The standard formula for TPFSC from Chapter 3 was used, while GSI and HSI are described below.

$$GSI = (GW / BW) \times 100$$

$$HSI = (HW / BW) \times 100$$

where,

GW = gonad's weight

HW = hepatopancreas' weight

BW = body weight

5.3.4. Statistical analyses

Statistical analyses and graphing were performed using custom scripts in RStudio version 4.1.2 (R Core Team, 2022 ; RStudio Inc., Boston, MA, USA). Diagnostic plots were employed to detect heterogeneity in variance and non-normal distribution of data residuals before the analysis. Log transformation was used on sperm concentration, GSI, and TPFSC data, while logit transformation was used on sperm viability and DNA fragmentation data to correct non-normality or heteroscedasticity before the analysis. The effect of the dietary treatment supplemented with AX, CHO, and AX + CHO and the length of time on treatment on different reproductive performance indicators was evaluated using analysis of variance (ANOVA). A pairwise comparison using the *glht* function was performed to determine differences among dietary treatments at Days 30 and 60. The raw data were used in plotting graphs, exploring relationships between covariates and response variables with significant relationships. The survival distribution of animals fed the different dietary treatments was evaluated using Cox regression. Differences between variables were considered significant at $p \leq 0.05$. Data variables were summarised as mean ± SEM.

5.4. Results

Among reproductive indicators, the gonadosomatic index (GSI) showed significant differences among the dietary treatments on Day 30 (Table 5.2). Compared to those fed the control diet, crayfish fed with AX ($p = 0.05$) or CHO ($p = 0.04$) supplemented diets had increased GSI at Day 30. However, other reproductive parameters were comparable between dietary treatments at Days 30 and 60 ($p > 0.05$).

Significant differences in some reproductive parameters were also found in association with the length of time crayfish were exposed to dietary treatments (Table 5.3). In particular, spermatozoa viability was significantly affected by the duration of exposure to all dietary treatments (Table 5.3). Compared to Day 0, mean sperm viability increased significantly by 20.8 % and 20.3 % after 30 and 60 days, respectively, for all diet formulations (Table 5.4). The mean value for sperm DNA fragmentation and concentration also significantly increased over the duration of feeding across all treatment groups (Table 5.3). However, these effects were not associated with a significant change in the potential fertile sperm concentration (TPFSC; Tables 5.3 & 5.4). Sperm DNA fragmentation increased by a mean of 22.8 % after 60 days of feeding the supplemented compared with the mean value at Day 0 (Table 5.4). The mean sperm concentration of crayfish in all treatment groups increased significantly by 22 % by Day 30, then decreased by 1.5 % by Day 60, which did not differ significantly from the control treatment on Day 0 (Tables 5.3 & 5.4).

The mean spermatophore weight increased significantly by 21.1 % and 41.9 % after 30 and 60 days, respectively (Tables 5.3 & 5.4; Figure 5.2 d). Significant increases were also

observed after 30 and 60 days for the mean GSI (18.8 % and 23.1 %, respectively) and mean HSI (10.4 % and 13.1 %, respectively; Tables 5.3 & 5.4; Figures 5.2 e – f).

Using Cox regression, no significant difference was found in the survival distribution over time for animals fed the different dietary treatments ($p = 0.788$). Survival rates of crayfish fed the control, AX, CHO, and AX + CHO dietary treatments were 93.9 %, 89.2 %, 87.5 %, and 93.9 %, respectively.

Table 5.2. Reproductive performance of male redclaw broodstock (*C. quadricarinatus*) subjected to four different dietary treatments at three different time points. Data are presented as mean ± SEM.

Response variables	Day 0	Day 30				Day 60				Time effect (Sig.)
		Control	AX	CHO	AX + CHO	Control	AX	CHO	AX + CHO	
Sperm viability (%)	77.5 ± 1.4	98.3 ± 2.0	98.0 ± 2.0	98.4 ± 2.0	98.6 ± 2.0	97.9 ± 2.2	98.0 ± 2.2	97.6 ± 2.2	97.8 ± 2.2	***
Sperm DNA fragmentation (%)	22.2 ± 3.4	-	-	-	-	45.9 ± 6.0	40.8 ± 6.0	49.4 ± 6.0	44.0 ± 6.0	***
Sperm concentration (x 10^4 cells/ml)	53.6 ± 5.9	62.4 ± 8.6	66.1 ± 8.6	69.9 ± 8.6	63.2 ± 8.6	51.0 ± 9.3	47.2 ± 9.3	53.5 ± 9.3	59.6 ± 9.3	**
Total potential sperm concentration (x 10^4 cells/ml)	33.5 ± 4.9	-	-	-	-	27.7 ± 7.8	29.8 ± 7.8	28.9 ± 7.8	34.9 ± 7.8	NS
Spermatophore weight (g)	0.031 ± 0.002	0.036 ± 0.003	0.036 ± 0.003	0.039 ± 0.003	0.036 ± 0.003	0.042 ± 0.004	0.042 ± 0.004	0.047 ± 0.004	0.043 ± 0.004	***
Gonadosomatic index (GSI)	0.56 ± 0.04	0.63 ± 0.06 [a]	0.78 ± 0.06 [b]	0.79 ± 0.06 [b]	0.71 ± 0.06 [ab]	0.74 ± 0.06	0.70 ± 0.06	0.73 ± 0.06	0.67 ± 0.06	***
Hepatosomatic index (HSI)	5.1 ± 0.2	6.1 ± 0.4	5.5 ± 0.3	5.7 ± 0.3	5.6 ± 0.3	5.9 ±0.3	6.0 ± 0.3	5.7 ± 0.3	5.7 ± 0.3	***

Different superscripts for each time point denote significant differences between dietary treatment means ($p \leq 0.05$).

NS - no significant differences.

** or *** as symbols for $p \leq 0.01$ and $p \leq 0.001$, respectively (*see Appendix A for the statistical summary*).

- means no data were analysed.

Table 5.3. Sperm quality indices and other reproductive potential parameters for all dietary treatments combined over the duration of feeding in male redclaw (*C. quadricarinatus*) broodstock. Data are presented as mean ± SEM.

Response variables	Duration of dietary treatment after acclimation (d)		
	0	30	60
Sperm viability (%)	77.5 ± 1.6 [a]	98.3 ± 1.6 [b]	97.8 ± 1.8 [b]
Sperm DNA fragmentation (%)	22.5 ± 2.6 [a]	-	45.4 ± 2.5 [b]
Sperm concentration (x 10^4 cells/ml)	53.6 ± 6.8 [a]	65.4 ± 3.2 [b]	52.8 ± 3.0 [a]
Total potential fertile sperm concentration (x 10^4 cells/ml)	33.5 ± 5.2	-	27.8 ± 8.2
Spermatophore weight (g)	0.031 ± 0.002 [a]	0.037 ± 0.001 [b]	0.043 ± 0.002 [b]
Gonadosomatic index (GSI)	0.58 ± 0.03 [a]	0.68 ± 0.03 [b]	0.71 ± 0.03 [b]
Hepatosomatic index (HSI)	5.1 ± 0.1 [a]	5.7 ± 0.1 [b]	5.9 ± 0.1 [b]

Different superscripts for each time period denote significant differences between treatment means ($p \leq 0.05$; *see Appendix A for the statistical summary*).

- means no data were analysed.

Table 5.4. Change in reproductive potential parameters over feeding duration after acclimation, including all dietary treatments combined in male redclaw (*C. quadricarinatus*) broodstock. Data are presented as mean ± SEM.

Response variables	Change from control at 0 d			
	30 d		60 d	
	Differences (se)	Sig.	Differences (se)	Sig.
Sperm viability (%)	20.8 (1.6)	***	20.3 (1.8)	***
Sperm DNA fragmentation (%)	-	-	22.8 (2.5)	***
Sperm concentration (x 10^4 cells/ml)	11.8 (4.4)	**	- 0.8 (4.8)	NS
Total potential fertile sperm concentration (x 10^4 cells/ml)	-	-	- 5.9 (8.2)	NS
Spermatophore weight (g)	0.008 (0.001)	***	0.015 (0.002)	***
Gonadosomatic index (GSI)	0.2 (0.03)	***	0.2 (0.03)	***
Hepatosomatic index (HSI)	0.5 (0.1)	***	0.7 (0.1)	***

Different superscripts for each time period denote significant differences between treatment means ($p \leq 0.05$).

NS - not significant.

** or *** as symbols for $p \leq 0.01$ and $p \leq 0.001$, respectively (*see Appendix A for the statistical summary*).

- means no data were analysed.

5.5. Discussion

In this study, reproductive parameters of redclaw male broodstock increased from Day 0 to Days 30 and 60, indicating that the animals were in the reproductive developmental stage during the course of the study. Dietary supplementation with AX or CHO accelerated the rate of gonad development to reproductive conditions within a 30-day window. The degree of DNA fragmentation in redclaw spermatozoa, however, doubled over the duration of the feeding trial, which could, perhaps, be related to other non-diet-related factors.

Dietary supplementation with AX or CHO accelerated gonad growth, as indicated by increased GSI, leading to faster maturation of male broodstock for reproduction. This results in improved reproductive parameters over 60 days, except for sperm DNA integrity and TPFSC. This growth could be attributed to the nutritional benefits of AX and CHO supplementation in the broodstock diet, which was found previously to be valuable in crustacean reproduction (Paibulkichakul et al., 2008; Guo et al., 2022). Astaxanthin was documented to protect cells, tissues and embryos from oxidative damage, while CHO acts as a precursor hormone that regulates moulting and reproduction in crustaceans (Liñán-Cabello et al., 2002; Kumar et al., 2018). In black tiger prawn (*Penaeus monodon*), gonadal maturation, fecundity, and spawning of female broodstock improved when fed with a diet supplemented with AX compared to an unsupplemented diet for 60 days (Pangantihon-Kühlmann et al., 1998; Paibulkichakul et al., 2008). In the same species, sperm count in male broodstock was greatly enhanced using an AX-supplemented diet (Paibulkichakul et al., 2008). The effect of AX in female redclaw (*C. quadricarinatus*) has been documented by Liñán-Cabello et al. (2004), who reported a 100 % survival; however, the oocyte maturation parameters were not significantly different between the control and treated groups 21 days

post-injection. In female crabs, supplementation with CHO in broodstock diets increased moulting rates and ovarian development by improving CHO transport and steroid hormone synthesis (Guo et al., 2022; Zhu et al., 2022a; Zhu et al., 2022b). In redclaw crayfish (*C. quadricarinatus*), most studies investigating the effects of dietary supplementation with CHO have assessed somatic growth indices and survival in juveniles. In contrast to Thompson et al. (2003), who reported that CHO supplementation provides no benefit, Hernández et al. (2004) found that 0.5% CHO supplementation improved juvenile growth in redclaw. In female juvenile freshwater crayfish (*Procambarus clarkii*), dietary supplementation with 10 % CHO enhanced growth, moulting and ovarian maturation (Hou et al., 2022). This study is the first report on the positive effect of dietary supplementation with AX, CHO, or both on reproductive potential in male redclaw broodstock.

After 60 days of dietary treatment, an increase in sperm concentration, viability and GSI were demonstrated. Aside from the dietary benefits, improvements in these reproductive potential parameters may have also been influenced by the maintenance of male broodstock in tanks within tightly regulated environmental conditions, including temperature and photoperiod, which are more favourable for breeding and gonadal maturation in redclaw crayfish (Jones et al., 1996; Bugnot and López Greco, 2009). In our study, wild crayfish were captured during winter (~ 15 °C) at sub-optimal temperatures for redclaw reproduction. After collection, male redclaw were maintained in breeding tanks with regulated temperatures (26.8 ± 0.1 °C) and photoperiods (14 L: 10 D) conducive to redclaw reproduction. These controlled environmental conditions are similar to the reported optimal ranges for gonadal maturation and spawning in redclaw females documented by Jones (1995a) and Barki et al. (1997). Reynolds (2002) documented that redclaw crayfish are summer brooders with a natural reproductive season from late spring to summer. This claim was supported by Bugnot and

López Greco (2009) after evaluating sperm count and viability in redclaw males in different seasons and at different temperatures. They showed that sperm count was greater in summer, with the peak number of sperm and viability found when temperatures were between 26.5 – 29.5 ºC. It would be interesting to investigate the effect of different photoperiods and complete darkness on sperm quality, given that complete darkness was documented to enhance gonadal maturation and spawning in female freshwater crayfish (Daniels et al., 1994; Harlioğlu and Duran, 2010; Liu et al., 2013).

The increase in mean spermatophore weight at Days 30 and 60, compared to Day 0, could be attributed to an increase in the GSI after exposure to a water temperature of around 27 ºC. This temperature favoured reproductive capacity compared to lower water temperatures that would have prevailed at the time of collection from the wild. Tropea et al. (2010) have reported that higher temperatures induce redclaw males to expend more energy towards gonadal development, resulting in a greater proportion of mature testicular lobes. These researchers observed an increase in secretory activity along the epithelium of the proximal and middle part of the vas deferens. They concluded that hypertrophied cytoplasmic droplets in the distal section of the vas deferens increase the stratification of the secondary spermatophore layer, and this occurrence may explain a heavier spermatophore in this study. In Chapter 3, the study demonstrated a significant association between spermatophore weight and sperm concentration. In this study, the significant increase in spermatophore weight on Day 30 is potentially attributed to the increase in sperm concentration. However, the reason for the decline in sperm concentration between Days 30 and 60 while the weight of spermatophores remained relatively constant during that time could not be determined and requires further investigation. Thus, changes to the primary or secondary layers of the

spermatophore and/or the volume of the spermatophore are more likely contributing to the increase in the weight of spermatophores that was observed.

In this study, sperm DNA fragmentation increased by 200 % after 60 days of feeding, irrespective of the diet used. This change occurred regardless of animals being maintained within optimal environmental conditions for reproduction. In the literature, factors that potentially contribute to increased sperm DNA damage in spermatozoa include the source of animals, inadequate levels of dietary antioxidants or essential nutrients, semen extender composition, changes in environmental conditions, and physiological stress. Feng et al. (2018), for example, reported that sperm DNA fragmentation, measured using the sperm chromatin dispersion test, in captive-bred black tiger prawns (*Penaeus monodon*; 6.8 ± 4.5 %; mean ± SD) was higher than that of prawns sourced from the wild (3.3 ± 1.5%; mean ± SD). In vertebrates, antioxidants in diets or semen extenders can reduce oxidative stress on sperm DNA by mitigating the adverse effects of ROS (Figueroa et al., 2018; Peña et al., 2019a). The use of antioxidants such as α-tocopherol and ascorbic acid in sperm freezing medium decreased lipid peroxidation and increased fertilisation rate (80 % – 90 %) in cryopreserved spermatozoa of Atlantic salmon (*Salmo salar*; Figueroa et al., 2018). Alternatively, supplementing boar diets with antioxidants reduced sperm DNA damage by 55 % under heat-stress conditions (Peña et al., 2019a). While the effect of changes in environmental temperature on sperm DNA integrity has not been evaluated in decapod crustaceans, heat stress has been reported to induce sperm DNA damage in vertebrates (Paul et al., 2008; Peña et al., 2017; Hamilton et al., 2018; Peña et al., 2019b; Abdollahi et al., 2021). For example, in boars, the sperm DNA fragmentation measured using a TUNEL assay during tropical summer (29.2 ± 0.2 ºC) showed 16-fold greater DNA damage than that of samples collected during winter (24.2 ± 0.4 ºC; Peña et al., 2019b). In economically important aquaculture

species, a relatively high value of 43.5 ± 6 % (mean ± SEM) sperm DNA fragmentation was observed in captive-bred barramundi (*Lates calcarifer*) when being maintained in breeding tanks with an optimum water temperature for reproduction of 30 ºC (Marc et al., 2021). Similarly, DNA fragmentation in domesticated whiteleg shrimp spermatozoa (*Litopenaeus vannamei*) averaged 20.7 ± 3.2 % (mean ± SEM; Duangjai et al., 2023) maintained in tanks with a water temperature of 27 – 29 °C. It appears that the documented water temperatures for these aquaculture commodities may impact the sperm DNA integrity of the broodstock, and the influence of optimum environmental conditions for reproduction on sperm DNA integrity requires further investigation. Possible effects of physiological stress, such as stocking density and effects of photoperiod on ovarian development, spawning rate and pleopodal egg number in female crayfish, have been reported (Barki and Karplus, 2000; Liu et al., 2013; Harlıoğlu and Farhadi, 2017). However, the effect on sperm quality, specifically DNA fragmentation, has not been investigated. The increase in DNA fragmentation in this study over the period of confinement is concerning, as it may have an adverse effect on fertility and warrants further investigation into potential causes of this deterioration and effects on fertility.

In aquaculture, the HSI is a biological index commonly employed as an indicator of energy status and reproductive maturation in finfishes (Nunes et al., 2011; Rizzo and Bazzoli, 2020) and crustaceans (Sánchez-Paz et al., 2007; Wang et al., 2014). HSI is functionally similar in finfish and crustaceans, and refers to the weight of the liver in finfish and the hepatopancreas in crustaceans expressed as a percentage of the total body weight (Cervellione et al., 2017). In crustaceans, the hepatopancreas is an organ with diverse functions responsible for nutrient absorption, intracellular digestion and energy storage that can be available when animals demand more energy or when feeding decreases (Vogt et al., 1989; Wang et al., 2014). The

potential energy available for gonadal maturation is increased with higher HSI (Abduh et al., 2021). In this study, a significant increase in HSI was observed after 30 and 60 days of dietary supplementation, but there was no significant effect of dietary treatment. Presumably, each diet produced a positive energy balance; increasing HSI may be due to excess nutrients beyond maintenance, growth and reproduction requirements. This claim can be further validated by conducting a biochemical composition analysis of the feed and hepatopancreas sample from each dietary treatment.

The broodstock diet formulated for this study resulted in high survival rates in crayfish fed each dietary treatment. Other studies reported survival rates in redclaw breeders of more than 83 % after 70 (Cortés-Jacinto et al., 2004) and 127 days (Jones, 1990). Similarly, our study reported more than 87 % survival rates in male redclaw breeders, suggesting that the developed plant-based diet could be suitable as a broodstock diet for redclaw crayfish. However, further investigation is recommended to assess the diet's impact on reproduction in a longer-term feeding trial.

5.6. Conclusion

Overall, this study demonstrated that dietary supplementation with AX or CHO accelerated gonadal maturity in male redclaw broodstock within 30 days of feeding, exhibiting an increase in GSI, spermatophore weight, and sperm quality parameters but not DNA integrity after 60 days. This finding indicates that male redclaw broodstock attains a reproductive condition within a month and can potentially be used for breeding and reproductive purposes, which could shorten sexual maturation within commercial aquaculture. However, further investigation is warranted to determine if additional dietary modifications can improve sperm

quality parameters, particularly DNA integrity. The improved HSI on Days 30 and 60 and high survival rates throughout the experiment indicate that the plant-based diet could be a baseline formulation for developing optimal broodstock diets in the future.

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5.8. References

Abdollahi, F., Farhang Dehghan, S., Amanpour, S., Haghparast, A., Sabour, S., Zendehdel, R., 2021. Effect of co-exposure to heat and psychological stressors on sperm DNA and semen parameters. Toxicol. Rep. 8, 1948-1954. https://doi.org/https://doi.org/10.1016/j.toxrep.2021.11.015.

Abduh, M.Y., Koh, I.C.C., Abol-Munafi, A.B., Norazmi-Lokman, N.H., Mat Noordin, N., 2021. Effects of dietary fish oil and corn oil on gonadosomatic and hepatosomatic index, gonadal histology, 17β-oestradiol level and fatty acids profile of mahseer (*Tor tambroides*) broodstock in captivity. Aquac. Nutr. 27(5), 1448-1459. https://doi.org/https://doi.org/10.1111/anu.13282.

Aquino, J.I.L., Elliott, L., Zeng, C., Paris, D.B.B.P., 2022. Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation, and their potential application to decapod crustacean aquaculture. Rev. Aquac. 14(2), 848-889. https://doi.org/https://doi.org/10.1111/raq.12627.

Barki, A., Karplus, I., 2000. Crowding female red claw crayfish, *Cherax quadricarinatus*, under small-tanks hatchery conditions: what is the limit? Aquaculture 181(3), 235-240. https://doi.org/https://doi.org/10.1016/S0044-8486(99)00235-5.

Barki, A., Levi, T., Hulata, G., Karplus, I., 1997. Annual cycle of spawning and molting in the red-claw crayfish, *Cherax quadricarinatus*, under laboratory conditions. Aquaculture 157(3), 239-249. https://doi.org/https://doi.org/10.1016/S0044-8486(97)00163-4.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165(3), 535-548. https://doi.org/https://doi.org/10.1016/j.ygcen.2009.02.011.

Bray, W.A., Lawrence, A.L., 1998. Male viability determinations in *Penaeus vannamei*: evaluation of short-term storage of spermatophores up to 36 h and comparison of Ca-free saline and seawater as sperm homogenate media. Aquaculture 160(1), 63-67. https://doi.org/https://doi.org/10.1016/S0044-8486(97)00225-1.

Bugnot, A.B., López Greco, L.S., 2009. Sperm production in the red claw crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). Aquaculture 295(3), 292-299. https://doi.org/https://doi.org/10.1016/j.aquaculture.2009.07.021.

Cabrita, E., Robles, V., Herráez, M.P., 2009. Sperm quality assessment, in: Cabrita, E., Robles, V., Herráez, M.P. (Eds.), Methods in Reproductive Aquaculture: Marine andFreshwater Species. Biology Series. CRC Press (Taylor and Francis Group), Boca Raton, Florida, USA, pp. 93-148.

Cervellione, F., McGurk, C., Van den Broeck, W., 2017. “Perigastric organ”: a replacement name for the “hepatopancreas” of Decapoda. J. Crustac. Biol. 37(3), 353-355. https://doi.org/10.1093/jcbiol/rux020.

Chen, C., Xu, C., Yang, X., Qian, D., Gu, Z., Jia, Y., Li, E., 2021. Growth, antioxidant capacity, intestine histology and lipid metabolism of juvenile red claw crayfish, *Cherax quadricarinatus*, fed different lipid sources. Aquac. Nutr. 27(1), 261-273. https://doi.org/https://doi.org/10.1111/anu.13183.

Cortés-Jacinto, E., Villarreal-Colmenares, H., Civera-Cerecedo, R., Cruz-Suárez, L.E., 2004. Studies on the nutrition of the freshwater crayfish *Cherax quadricarinatus* (von Martens): effect of the dietary protein level on growth of juveniles and preadults. Freshw. Crayfish 14, 70 - 80. doi: not available.

Cortés-Jacinto, E., Villarreal-Colmenares, H., Civera-Cerecedo, R., Martínez-Córdova, R., 2003. Effect of dietary protein level on growth and survival of juvenile freshwater crayfish *Cherax quadricarinatus* (Decapoda: Parastacidae). Aquac. Nutr. 9(4), 207-213. https://doi.org/https://doi.org/10.1046/j.1365-2095.2003.00241.x.

Cortés-Jacinto, E., Villarreal-Colmenares, H., Civera-Cerecedo, R., Naranjo-Páramo, J., 2004. Effect of dietary protein level on the growth and survival of pre-adult

freshwater crayfish *Cherax quadricarinatus* (von Martens) in monosex culture. Aquac. Res. 35(1), 71-79. https://doi.org/https://doi.org/10.1111/j.1365-2109.2004.00988.x.

Cortés-Jacinto, E., Villarreal-Colmenares, H., Cruz-Suarez, L.E., Civera-Cerecedo, R., Nolasco-Soria, H., Hernandez-Llamas, A., 2005. Effect of different dietary protein and lipid levels on growth and survival of juvenile Australian redclaw crayfish, *Cherax quadricarinatus* (von Martens). Aquac. Nutr. 11(4), 283-291. https://doi.org/https://doi.org/10.1111/j.1365-2095.2005.00353.x.

Daniels, W.H., D'Abramo, L.R., Graves, K.F., 1994. Ovarian development of female red swamp crayfish (*Procambarus clarkii*) as influenced by temperature and photoperiod. J. Crustac. Biol. 14(3), 530-537. https://doi.org/10.1163/193724094x00100.

Duangjai, E., Umyoo, P., Meunpol, O., Puanglarp, N., 2023. Sperm quality assessment of white shrimp (*Litopenaeus vannamei*) broodstock using comet assay. Trends Sci. 20(6), 4768. https://doi.org/10.48048/tis.2023.4768.

Félix, F., Oliveira, C.C.V., Cabrita, E., 2021. Antioxidants in fish sperm and the potential role of melatonin. Antioxidants 10(1), 1 - 36. https://doi.org/https://doi.org/10.3390/antiox10010036.

Feng, T., 2018. Spermatogenesis and Sperm Assessment in the Black Tiger Prawn, *Penaeus monodon*, School of Agriculture and Food Sciences. University of Queensland, Queensland, Australia, p. 195.

Feng, T., Gosálvez, J., Lopez-Fernandez, C., Arroyo, F., Paterson, B., Johnston, S., 2018. Sperm chromatin dispersion test (SCDt) for the assessment of sperm DNA fragmentation in black tiger prawn, *Penaeus monodon*. Aquaculture 491, : 281-288. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.03.041.

Feng, T., Paterson, B., Coman, G., Johnston, S., 2019. Evaluating the impact of moulting and chilled storage of spermatophores on the integrity of plasma membrane, acrosome and DNA of black tiger prawn (*Penaeus monodon*) spermatozoa. Aquac. Res. 50(1), 226-235. https://doi.org/https://doi.org/10.1111/are.13888.

Figueroa, E., Farias, J.G., Lee-Estevez, M., Valdebenito, I., Risopatrón, J., Magnotti, C., Romero, J., Watanabe, I., Oliveira, R.P.S., 2018. Sperm cryopreservation with supplementation of α-tocopherol and ascorbic acid in freezing media increase sperm function and fertility rate in Atlantic salmon (*Salmo salar*). Aquaculture 493, : 1-8. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.04.046.

Guo, H., Wang, M., Wang, X., Xiao, K., Huang, Y., Hua, H., Xiong, W., Liu, W., Abasubong, K.P., Qiang, W., Chi, C., Jiang, G., 2022. Effect of dietary cholesterol on ovarian development of Chinese mitten crabs (*Eriocheir sinensis*). Front. Mar. Sci. 9. https://doi.org/10.3389/fmars.2022.1070829.

Hamilton, T., Siqueira, A.F.P., de Castro, L.S., Mendes, C.M., Delgado, J.C., de Assis, P.M., Mesquita, L.P., Maiorka, P.C., Nichi, M., Goissis, M.D., Visintin, J.A., Assumpção, M., 2018. Effect of heat stress on sperm DNA: Protamine assessment in ram spermatozoa and testicle. Oxid. Med. Cell Longev. 2018, 5413056. https://doi.org/10.1155/2018/5413056.

Harlioğlu, M.M., Duran, T.Ç., 2010. The effect of darkness on mating and pleopodal egg production time in a freshwater crayfish, *Astacus leptodactylus* Eschscholtz. Aquac. Int. 18(5), 843-849. https://doi.org/10.1007/s10499-009-9305-z.

Harlıoğlu, M.M., Farhadi, A., 2017. Factors affecting the reproductive efficiency in crayfish: implications for aquaculture. Aquac. Res. 48(5), 1983-1997. https://doi.org/https://doi.org/10.1111/are.13263.

Harlioğlu, M.M., Köprücü, K., Harlioğlu, A.G., Yonar, S.M., Duran, T.Ç., Çakmak, M.N., Aksu, Ö., Özcan, S., Kutluyer, F., Gündoğdu, H., 2013. Effect of dietary n-3 series fatty acids on sperm production in the freshwater crayfish, *Astacus leptodactylus* (Eschscholtz) (Astacidae). Aquac. Int. 21(2), 273-282. https://doi.org/https://doi.org/10.1007/s10499-012-9549-x.

Harrison, K.F., 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: A review. J. Shellfish Res. 2(1), 1 - 23.

Hernández, P.V., Olvera-Novoa, M.A., Rouse, D.B., 2004. Effect of dietary cholesterol on growth and survival of juvenile redclaw crayfish *Cherax quadricarinatus* under laboratory conditions. Aquaculture 236(1), 405-411. https://doi.org/https://doi.org/10.1016/j.aquaculture.2003.12.005.

Hou, S., Zhu, S., Li, J., Huang, J., Li, J., Cheng, Y., 2022. Effects of dietary phospholipid and cholesterol levels on growth, moulting performance, and ovary development in female juvenile crayfish (*Procambarus clarkii*). Aquac. Nutr. 2022, 4033033. https://doi.org/10.1155/2022/4033033.

Izquierdo, M.S., Fernández-Palacios, H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197(1), 25-42. https://doi.org/https://doi.org/10.1016/S0044-8486(01)00581-6.

Jones, C.M., 1990. The biology and aquaculture potential of the tropical freshwater crayfish, *Cherax quadricarinatus*. Department of Primary Industries Queensland, Brisbane, QLD, Australia.

Jones, C.M., 1995a. Production of juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae) I. Development of hatchery and nursery procedures. Aquaculture 138(1), 221-238. https://doi.org/https://doi.org/10.1016/0044-8486(95)00068-2.

Jones, C.M., 1995b. Production of juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae) II. Juvenile nutrition and habitat. Aquaculture 138(1), 239-245. https://doi.org/https://doi.org/10.1016/0044-8486(95)00069-0.

Jones, C.M., Ruscoe, I.M., Queensland, F.F.a.A.C., 1996. Production technology for redclaw crayfish (*Cherax quadricarinatus*): Final report. Freshwater Fisheries and Aquaculture Centre, Department of Primary Industries, Walkamin, QLD.

Jones, C.M., Valverde, C., 2020. Development of mass production hatchery technology for the redclaw crayfish, *Cherax quadricarinatus*. Freshw. Crayfish 25(1), 1 - 6. https://doi.org/10.5869/fc.2020.v25-1.001.

Kumar, V., Sinha, A.K., Romano, N., Allen, K.M., Bowman, B.A., Thompson, K.R., Tidwell, J.H., 2018. Metabolism and nutritive role of cholesterol in the growth, gonadal development, and reproduction of crustaceans. Rev. Fish. Sci. Aquac. 26(2), 254-273. https://doi.org/10.1080/23308249.2018.1429384.

Leelatanawit, R., Uawisetwathana, U., Khudet, J., Klanchui, A., Phomklad, S., Wongtripop, S., Angthoung, P., Jiravanichpaisal, P., Karoonuthaisiri, N., 2014. Effects of polychaetes (*Perinereis nuntia*) on sperm performance of the domesticated black tiger shrimp (*Penaeus monodon*). Aquaculture 433, : 266-275. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.06.034.

Leung-Trujillo, J.R., Lawrence, A.L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65(3), 363-370. https://doi.org/https://doi.org/10.1016/0044-8486(87)90249-3.

Lim, K.C., Yusoff, F.M., Shariff, M., Kamarudin, M.S., 2018. Astaxanthin as feed supplement in aquatic animals. Rev. Aquac. 10(3), 738-773. https://doi.org/https://doi.org/10.1111/raq.12200.

Liñán-Cabello, M.A., Medina-Zendejas, R., Sánchez-Barajas, M., Mena Herrera, A., 2004. Effects of carotenoids and retinol in oocyte maturation of crayfish *Cherax quadrucarinatus*. Aquac. Res. 35(9), 905-911. https://doi.org/10.1111/j.1365-2109.2004.01083.x.

Liñán-Cabello, M.A., Paniagua-Michel, J., Hopkins, P.M., 2002. Bioactive roles of carotenoids and retinoids in crustaceans. Aquac. Nutr. 8(4), 299-309. https://doi.org/10.1046/j.1365-2095.2002.00221.x.

Liu, S., Gong, S., Li, J., Huang, W., 2013. Effects of water temperature, photoperiod, eyestalk ablation, and non-hormonal treatments on spawning of ovary-mature red swamp crayfish. N. Am. J. Aquac. 75(2), 228-234. https://doi.org/10.1080/15222055.2012.746247.

López-Greco, L.S.S., Liane, Timpanaro, S., Cid, A.R., Lamberti, M., Battista, A., LauraTomas, A., Jones, C.M., 2022. Impact of low-cost diets on maturation of the red

claw crayfish *Cherax quadricarinatus:* An integrative approach during a long-term study. Aquaculture 561, 738614. https://doi.org/https://doi.org/10.1016/j.aquaculture.2022.738614.

Lu, X., Peng, D., Chen, X., Wu, F., Jiang, M., Tian, J., Liu, W., Yu, L., Wen, H., Wei, K., 2020. Effects of dietary protein levels on growth, muscle composition, digestive enzymes activities, hemolymph biochemical indices and ovary development of pre-adult red swamp crayfish (*Procambarus clarkii*). Aquac. Rep. 18, 100542. https://doi.org/https://doi.org/10.1016/j.aqrep.2020.100542.

Medley, P.B.C., Jones, C.M., W. Jr Avault, J., 1994. A global perspective of the culture of Australian redclaw crayfish, *Cherax quadricarinatus*: Production, economics and marketing. J. World Aquac. Soc. 25(4), 6-13.

Méndez-Martínez, Y., Ceseña, C.E., Luna-González, A., García-Guerrero, M.U., Martinez-Porchas, M., Campa-Cordova, Á.I., Cortés-Jacinto, E., 2021. Effects of different dietary protein-energy ratios on growth, carcass amino acid and fatty acid profile of male and female *Cherax quadricarinatus* (von Martens, 1868) pre-adults. Aquac. Nutr. 27(6), 2481-2496. https://doi.org/https://doi.org/10.1111/anu.13379.

Meyers, S.P., Latscha, T., 1997. Carotenoids, in: D'Abramo, L.R., Conklin, D.E., Akiyama, D.M. (Eds.), Crustacean Nutrition Advances in World Aquaculture, World Aquaculture Society, Baton Rouge, LA, pp. 164 - 193.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Rev. Aquac. 5(s1), S194-S223. https://doi.org/https://doi.org/10.1111/raq.12025.

Miki, W., 1991. Biological functions and activities of animal carotenoids. Pure Appl. Chem 63(1), 141-146. https://doi.org/doi:10.1351/pac199163010141.

Nunes, C., Silva, A., Soares, E., Ganias, K., 2011. The use of hepatic and somatic indices and histological information to characterize the reproductive dynamics of Atlantic sardine *Sardina pilchardus* from the Portuguese Coast. Mar. Coast. Fish. 3(1), 127-144. https://doi.org/https://doi.org/10.1080/19425120.2011.556911.

Paibulkichakul, C., Piyatiratitivorakul, S., Sorgeloos, P., Menasveta, P., 2008. Improved maturation of pond-reared, black tiger shrimp (*Penaeus monodon*) using fish oil and astaxanthin feed supplements. Aquaculture 282(1), 83-89. https://doi.org/https://doi.org/10.1016/j.aquaculture.2008.06.006.

Pangantihon-Kühlmann, M.P., Millamena, O., Chern, Y., 1998. Effect of dietary astaxanthin and vitamin A on the reproductive performance of *Penaeus monodon* broodstock. Aquat. Living Resour. 11(6), 403-409. https://doi.org/https://doi.org/10.1016/S0990-7440(99)80006-0.

Parnes, S., Sagi, A., 2002. Intensification of redclaw crayfish *Cherax quadricarinatus* culture. I. Hatchery and nursery system. Aquac. Eng. 26(4), 251-262. https://doi.org/https://doi.org/10.1016/S0144-8609(02)00034-1.

Paul, C., Murray, A.A., Spears, N., Saunders, P.T.K., 2008. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. Reproduction 136(1), 73-84. https://doi.org/https://doi.org/10.1530/rep-08-0036.

Pavasovic, A., Anderson, A.J., Mather, P.B., Richardson, N.A., 2007a. Effect of a variety of animal, plant and single cell-based feed ingredients on diet digestibility and digestive enzyme activity in redclaw crayfish, *Cherax quadricarinatus* (Von Martens 1868). Aquaculture 272(1), 564-572. https://doi.org/https://doi.org/10.1016/j.aquaculture.2007.08.027.

Pavasovic, A., Anderson, A.J., Mather, P.B., Richardson, N.A., 2007b. Influence of dietary protein on digestive enzyme activity, growth and tail muscle composition in redclaw crayfish, *Cherax quadricarinatus* (von Martens). Aquac. Res. 38(6), 644-652. https://doi.org/https://doi.org/10.1111/j.1365-2109.2007.01708.x.

Pavasovic, A., Richardson, N.A., Mather, P.B., Anderson, A.J., 2006. Influence of insoluble dietary cellulose on digestive enzyme activity, feed digestibility and survival in the red claw crayfish, *Cherax quadricarinatus* (von Martens). Aquac. Res. 37(1), 25-32. https://doi.org/https://doi.org/10.1111/j.1365-2109.2005.01389.x.

Peña, S.T., Jr., Gummow, B., Parker, A.J., Paris, D.B.B.P., 2019a. Antioxidant supplementation mitigates DNA damage in boar (*Sus scrofa domesticus*) spermatozoa induced by tropical summer. *PLoS One* 14(4), e0216143. https://doi.org/https://doi.org/10.1371/journal.pone.0216143.

Peña, S.T., Jr., Stone, F., Gummow, B., Parker, A.J., Paris, D.B.B.P., 2019b. Tropical summer induces DNA fragmentation in boar spermatozoa: implications for evaluating seasonal infertility. Reprod. Fertil. Dev. 31(3), 590-601. https://doi.org/https://doi.org/10.1071/RD18159.

Pérez-Cerezales, S., Martínez-Páramo, S., Beirão, J., Herráez, M.P., 2010. Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 139(6), 989. https://doi.org/10.1530/rep-10-0037.

Pérez-Rodríguez, J.C., Gómez-Gutiérrez, J., López-Greco, L.S., Cortés-Jacinto, E., 2019. Spermatophore production and sperm quality of the river prawn *Macrobrachium americanum* Spence Bate, 1868 fed with different diets. Aquac. Res. 0(0), 1-13. https://doi.org/https://doi.org/10.1111/are.14265.

R.Core.Team, 2022. R: A language and environment for statistical computing, R Foundation for Statistical Computing. Vienna, Austria.

Reynolds, J.D., 2002. Growth and Reproduction, in: Holdich, D.M. (Ed.), Biology of Freshwater Crayfish. Blackwell Science, Oxford, pp. 152–191.

Riesco, M.F., Valcarce, D.G., Martínez-Vázquez, J.M., Robles, V., 2019. Effect of low sperm quality on progeny: a study on zebrafish as model species. Sci. Rep. 9(1), 11192. https://doi.org/10.1038/s41598-019-47702-7.

Rigg, D., Seymour, J., Courtney, R., Jones, C., 2020. A review of juvenile redclaw crayfish *Cherax quadricarinatus* (von Martens, 1898) production in aquaculture. Freshw. Crayfish 25(1), 13-30. https://doi.org/10.5869/fc.2020.v25-1.013.

Rizzo, E., Bazzoli, N., 2020. Chapter 13 - Reproduction and embryogenesis, in: Baldisserotto, B., Urbinati, E.C., Cyrino, J.E.P. (Eds.), Biology and physiology of freshwater neotropical fish. Academic Press, pp. 287-313.

Samuel, M.J., Kannupandi, T., Soundarapandian, P., 1999. Nutritional effects on male reproductive performance in the freshwater prawn *Macrobrachium malcolmsonii* (H. Milne Edwards). Aquaculture 172(3), 327-333. https://doi.org/https://doi.org/10.1016/S0044-8486(98)00500-6.

Sánchez-Paz, A., García-Carreño, F., Hernández-López, J., Muhlia-Almazán, A., Yepiz-Plascencia, G., 2007. Effect of short-term starvation on hepatopancreas and plasma energy reserves of the Pacific white shrimp (*Litopenaeus vannamei*). J. Exp. Mar. Biol. Ecol. 340(2), 184-193. https://doi.org/https://doi.org/10.1016/j.jembe.2006.09.006.

Saoud, I.P., Ghanawi, J., Thompson, K.R., Webster, C.D., 2013. A review of the culture and diseases of redclaw crayfish *Cherax quadricarinatus* (Von Martens 1868). J. World Aquac. Soc. 44(1), 1-29. https://doi.org/10.1111/jwas.12011.

Shehata, A.I., Alhoshy, M., Wang, T., Mohsin, M., Wang, J., Wang, X., Han, T., Wang, Y., Zhang, Z., 2023. Dietary supplementations modulate the physiological parameters, fatty acids profile and the growth of red claw crayfish (*Cherax quadricarinatus*). J. Anim. Physiol. Anim. Nutr. (Berl.) 107(1), 308-328. https://doi.org/https://doi.org/10.1111/jpn.13704.

Simon, L., Murphy, K., Shamsi, M.B., Liu, L., Emery, B., Aston, K.I., Hotaling, J., Carrell, D.T., 2014. Paternal influence of sperm DNA integrity on early embryonic development. Hum. Reprod. 29(11), 2402-2412. https://doi.org/https://doi.org/10.1093/humrep/deu228.

Stumpf, L., Calvo, N.S., Pietrokovsky, S., López Greco, L.S., 2010. Nutritional vulnerability and compensatory growth in early juveniles of the "red claw" crayfish *Cherax quadricarinatus*. Aquaculture 304(1), 34-41. https://doi.org/https://doi.org/10.1016/j.aquaculture.2010.03.011.

Stumpf, L., Sarmiento Cárdenas, P.N., Timpanaro, S., López Greco, L., 2019. Feasibility of compensatory growth in early juveniles of "red claw" crayfish *Cherax quadricarinatus* under high density conditions. Aquaculture 510, 302-310. https://doi.org/https://doi.org/10.1016/j.aquaculture.2019.05.053.

Stumpf, L., Tropea, C., López Greco, L.S., 2014. Recovery growth of *Cherax quadricarinatus* juveniles fed on two high-protein diets: Effect of daily feeding following a cyclic feeding period on growth, biochemical composition and activity of digestive enzymes. Aquaculture 433, 404-410. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.06.037.

Sugiani, D., Lusiastuti, A.M., Taukhid, nbsp, Purwaningsih, U., 2015. Treatments for *Temnocephalids Ectosymbiont Craspedella sp.* on *Cherax quadricarinatus* and *Cherax albertisii* (Papua Freshwater Lobster). World J. Eng. Tech. 3(4), 48 - 54. https://doi.org/10.4236/wjet.2015.34C006.

Teshima, S., 1997. Phospholipids and sterols, in: D'Abramo, L.R., Conklin, D.E., Akiyama, D.M. (Eds.), Crustacean Nutrition. Advances in World Aquaculture, World Aquaculture Society, Baton Rouge, LA, pp. 85 - 107.

Thompson, K.R., Muzinic, L.A., Christian, T.D., Webster, C.D., Rouse, D.B., Manomaitis, L., 2003. Effect on growth, survival, and fatty acid composition of Australian red claw crayfish *Cherax quadricarinatus* fed practical diets with and without supplemental lecithin and/or cholesterol. J. World Aquac. Soc. 34(1), 1-10. https://doi.org/https://doi.org/10.1111/j.1749-7345.2003.tb00033.x.

Thompson, K.R., Muzinic, L.A., Engler, L.S., Webster, C.D., 2005. Evaluation of practical diets containing different protein levels, with or without fish meal, for juvenile Australian red claw crayfish (*Cherax quadricarinatus*). Aquaculture 244(1), 241-249. https://doi.org/https://doi.org/10.1016/j.aquaculture.2004.11.018.

Tropea, C., Piazza, Y., Greco, L.S.L., 2010. Effect of long-term exposure to high temperature on survival, growth and reproductive parameters of the "redclaw" crayfish *Cherax quadricarinatus*. Aquaculture 302(1), 49-56. https://doi.org/https://doi.org/10.1016/j.aquaculture.2010.01.027.

Vogt, G., Stöcker, W., Storch, V., Zwilling, R., 1989. Biosynthesis of *Astacus* protease, a digestive enzyme from crayfish. Histochemistry 91(5), 373-381. https://doi.org/10.1007/BF00493824.

Volkoff, H., London, S., 2018. Nutrition and reproduction in fish, in: Skinner, M.K. (Ed.), Encyclopedia of Reproduction (Second Edition). Academic Press, Oxford, pp. 743-748.

Vuthiphandchai, V., Nimrat, S., Kotcharat, S., Bart, A.N., 2007. Development of a cryopreservation protocol for long-term storage of black tiger shrimp (*Penaeus*

monodon) spermatophores. Theriogenology 68(8), 1192-1199. https://doi.org/https://doi.org/10.1016/j.theriogenology.2007.08.024.

Wagner, H., Cheng, J.W., Ko, E.Y., 2018. Role of reactive oxygen species in male infertility: An updated review of literature. Arab J. Urol. 16(1), 35-43. https://doi.org/https://doi.org/10.1016/j.aju.2017.11.001.

Wang, W., Wu, X., Liu, Z., Zheng, H., Cheng, Y., 2014. Insights into hepatopancreatic functions for nutrition metabolism and ovarian development in the crab *Portunus trituberculatus*: gene discovery in the comparative transcriptome of different hepatopancreas stages. PLoS One 9(1), e84921. https://doi.org/10.1371/journal.pone.0084921.

Xu, X.H., Xu, X.H., Yan, B.L., Xu, X.H., Yan, B.L., Peng, D.S., 2014. Comparative study on the acrosin activity in sperm of the marine crab *Charybdis japonica* (A. Milne-Edwards, 1861) (Brachyura, Portunidae) under different environmental conditions. Crustaceana 87(13), 1473-1485. https://doi.org/https://doi.org/10.1163/15685403-00003369.

Zenteno-Savín, T., Cortes-Jacinto, E., Vázquez-Medina, J.P., Villarreal-Colmenares, H., 2008. Oxidative damage in tissues of juvenile crayfish (*Cherax quadricarinatus* von Martens, 1868) fed with different levels of proteins and lipid. Hidrobiologica 18(2), 147-154.

Zhu, T., Jin, M., Xie, S., Guo, C., Luo, J., Zhang, X., Shen, Y., Sun, P., Jiao, L., Zhou, Q., 2022a. Transcriptome and targeted metabolomics revealed that cholesterol nutrition promotes ovarian development by regulating steroid hormone metabolism in swimming crab. Aquac. Rep. 27, 101396. https://doi.org/https://doi.org/10.1016/j.aqrep.2022.101396.

Zhu, T., Zhou, Q., Yang, Z., Zhang, Y., Luo, J., Zhang, X., Shen, Y., Jiao, L., Tocher, D.R., Jin, M., 2022b. Dietary cholesterol promotes growth and ecdysone signalling pathway by modulating cholesterol transport in swimming crabs (*Portunus trituberculatus*). Anim. Nutr. 10, 249-260. https://doi.org/https://doi.org/10.1016/j.aninu.2022.05.001.

Chapter 6. General discussion, conclusions, and future directions

6.1. General discussion

The demand for seafood for the growing global population has led to the search for more aquaculture commodities to address food security and sustainability (FAO, 2022). Crustaceans contribute to almost 30 % of global aquaculture production, and amongst freshwater crustaceans, crayfish are the most farmed and profitable (FAO, 2022). Redclaw crayfish (*Cherax quadricarinatus*) have a high potential as an aquaculture commodity for large-scale farming, but the inconsistent supply of early juveniles remains a bottleneck for the industry (Jones and Valverde, 2020; Rigg et al., 2020). Previous studies highlight the significant role of broodstock fertility in the early development and survival of fish larvae (Bobe and Labbé, 2010; Migaud et al., 2013; Kowalski and Cejko, 2019; Riesco et al., 2019). This may also be the case in redclaw crayfish, where subfertility issues contribute to fluctuating juvenile production. Evidence is increasing that poor male reproductive potential, detected by evaluating sperm quality, can result in embryonic deformities, lower offspring survival rates, and negative impacts on larval production in finfish (Pérez-Cerezales et al., 2010; Devaux et al., 2011; Riesco et al., 2019). The reproductive potential of male redclaw broodstock and its paternal role in fertilisation and subsequent embryonic development has never been reported and is poorly understood. The first step in understanding the impact of sperm quality on fertilisation and larval development is to establish a reliable quality measure. Hence, the overall objective of this thesis was to investigate the mechanisms of quantifying and optimising sperm quality for redclaw crayfish broodstock.

In Chapter 2, the literature on advanced reproductive techniques in decapod crustaceans was comprehensively reviewed (Aquino et al., 2022). Based on the information gathered, a set of feasible diagnostic tools were recommended to assess quality in redclaw spermatozoa and employed in each experimental chapter.

In Chapter 3, the reproductive potential of wild-caught male redclaw crayfish was quantified using conventional and advanced sperm quality diagnostic tools - the first time this has been reported. Important findings of this chapter included the detection of a high rate of sperm DNA fragmentation and low sperm viability in male redclaw broodstock. In addition, it was observed that heavier animals tended to have lesser sperm viability. Based on these findings, it appears male redclaw broodstock sourced from the wild potentially have sub-optimal fertility when initially collected at that time of year. Further study was needed to determine if a period of acclimation could be used to improve sperm quality prior to use in breeding programs. It was also evident that sperm quality varies significantly among males, however, results revealed that heavier spermatophores contain a greater sperm concentration, lesser sperm DNA damage, and enhanced concentration of potentially fertile spermatozoa. As a result, it is proposed that spermatophore weight is a useful measure for early assessment of sperm quality. The sperm quality diagnostic tools developed for redclaw can be helpful in screening highly fertile individuals for selective breeding, especially when broodstock are collected from the wild. In this chapter, we presented the first quantitative analysis of sperm morphology and identified a previously unreported putative tail-like structure in some redclaw spermatozoa. The presence of this structure warrants further investigation to determine its role in redclaw fertilisation and reproduction.

In Chapter 4, electroejaculation was tested as an alternative and less invasive method for collecting spermatophores from redclaw males in Experiment 1. Results demonstrated that electroejaculation is feasible in collecting spermatophores from wild-caught redclaw crayfish. The success rate of collection was 15 – 30 % in crayfish weighing between 60 to 130 g; success declined outside of this range. Experiment 2 compared the sperm quality in spermatophores collected by dissection and electroejaculation, which was previously unreported. Results revealed that removing spermatophores through dissection resulted in 38.6 % less sperm DNA fragmentation and over twice the concentration of potentially fertile spermatozoa compared to spermatophores collected by electroejaculation. It is suggested that redclaw spermatozoa recovered from spermatophores isolated via dissection could be preferable for use in future selective breeding programs that utilise cryopreservation and artificial fertilisation. However, male broodstock must be sacrificed to collect spermatozoa via dissection. In contrast, electroejaculation allows for repeated collection of spermatophores to assess the sperm quality of male broodstock intended for routine breeding and production. Poor sperm quality following electroejaculation may stem from physiological stress experienced by the animals, and further research is needed to confirm this hypothesis.

After establishing baseline reproductive data using adapted diagnostic tools for redclaw sperm quality (Chapter 3) and spermatophore collection (Chapter 4) protocols, in Chapter 5 results were reported on the effects of dietary treatments supplemented with astaxanthin (AX) and/or cholesterol (CHO), and the duration on dietary treatment on sperm quality and other reproductive performance indicators in redclaw male broodstock in a 60-day feeding trial. Broodstock nutrition is one of the controllable factors in the hatchery that could be modified to possibly enhance the poor sperm quality detected in redclaw in Chapter 3. Results showed that feeding redclaw crayfish with a plant-based diet supplemented with AX or CHO

accelerated gonadal maturation within 30-day timeframe, indicated by increased GSI, leading to faster development of male broodstock for reproduction. This improvement was observed in parameters such as sperm concentration, viability, and spermatophore weight. However, sperm DNA integrity showed no significant improvement, instead displaying a 200 % increase in DNA fragmentation, which could, perhaps, be related to other non-diet-related factors. The feeding trial resulted in high survival rates and HSI, suggesting that the culture, environmental conditions, and formulated diet were suitable for male redclaw broodstock; hence the plant-based diet used for the broodstock could serve as a useful reference for developing optimal future formulations.

6.2 Future directions

Further research is necessary to fully comprehend the impact of sperm quality in redclaw intensive breeding and reproduction. In this thesis, a method using a calcium-free saline solution was developed to hold spermatozoa post-extraction; however, more research is required to understand the impact of temperature, incubation time, media, supplements, and other extenders on sperm quality, including sperm DNA integrity and viability. To establish baseline data on the reproductive capacity of wild-caught male redclaw, it is valuable to investigate sperm quality throughout the breeding season. In addition, it would be worthwhile to assess the androgenic gland hormone, which is responsible for regulating spermatogenesis in redclaw crayfish, and its potential correlation with sperm quality. This study could contribute to a better understanding of redclaw male reproduction and developing more effective management and breeding strategies. The electroejaculation protocol in this study could be improved by investigating differences in electrical settings when collecting spermatophores from redclaw males. Further investigation is also needed to determine the

optimal time interval for regenerating spermatophores between repeated attempts of electroejaculation.

Aside from dietary factors, variables affecting sperm DNA integrity need more investigations, including effects of age, duration of acclimation, housing arrangements, stocking densities, presence of females, water temperature, and differences between captive-bred and wild-caught broodstock. The use of sperm quality diagnostic tools and the electroejaculation protocol in this study could be extended to other species, such as the critically endangered Hairy marron (*Cherax tenuimanus*; Duffy et al., 2014). These tools can further assess sperm quality in response to environmental, dietary or husbandry changes and are used in different protocols for sperm cryopreservation, spermatophore transport, and artificial fertilisation to accelerate selective breeding and genetic management of commercial redclaw aquaculture.

6.3. Limitation of the study

The scope of this thesis is confined to the use of the sperm quality diagnostic tools developed in this study, including concentration, viability, and DNA fragmentation. Further optimisation is required to develop tools for determining the energy and acrosome status of redclaw spermatozoa to provide additional information on the fertilising potential of redclaw spermatozoa. To verify sperm quality measured by these tools, a fertility trial could be conducted in which males with varying sperm quality breed with females to assess fertilisation and larval outcomes. Additionally, the electroejaculation protocol applied in this study was previously only tested on freshwater yabby (*C. destructor*; Jerry, 2001), and further investigation is needed to determine whether this method or other electrical stimulation

parameters could be applied to other *Cherax* and decapod species at different body weights. In Chapter 5, DNA fragmentation in redclaw spermatozoa on Day 30 was not measured due to insufficient reagents, hence the terminal samples were measured. In addition, the results were interpreted solely in terms of the reproductive performance of male redclaw broodstock. Further research is necessary to determine how the nutrient composition of each diet could affect sperm quality. This can be achieved by determining the biochemical compositions of the dietary treatments, gonads, and hepatopancreas.

6.4. Conclusions

In summary, the findings presented in this thesis provide producers and researchers with a better understanding of ways to extract, isolate and assess the quality of redclaw spermatozoa. The techniques developed can be applied to live animals to potentially screen males for fertility prior to use or to assess sperm quality for selective breeding purposes. This thesis demonstrated that redclaw males caught in the wild initially have poor reproductive potential. Among the controlled variables in the hatchery, nutrition is a key element that directly impacts reproductive performance, including sperm quality, in male redclaw, as shown in Chapter 5. The nutritional supplementation of astaxanthin (AX) or cholesterol (CHO) led to faster gonadal maturation, followed by improvement of other male reproductive indicators. Still, a two-fold increase in sperm DNA fragmentation was observed. Further investigation is necessary to determine whether dietary modifications, including optimisation of AX and CHO levels, could enhance sperm DNA integrity, identify potential causes of DNA damage and understand its effects on redclaw fertility.

6.5. References

Aquino, J.I.L., Elliott, L., Zeng, C., Paris, D.B.B.P., 2022. Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation, and their potential application to decapod crustacean aquaculture. Rev. Aquac. 14(2), 848-889. https://doi.org/https://doi.org/10.1111/raq.12627.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165(3), 535-548. https://doi.org/https://doi.org/10.1016/j.ygcen.2009.02.011.

Devaux, A., Fiat, L., Gillet, C., Bony, S., 2011. Reproduction impairment following paternal genotoxin exposure in brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*). Aquat. Toxicol. 101(2), 405-411. https://doi.org/https://doi.org/10.1016/j.aquatox.2010.11.017.

Duffy, R., Ledger, J., Dias, J., Snow, M., 2014. The critically endangered hairy marron, *Cherax tenuimanus* Smith, 1912: A review of current knowledge and actions required to prevent extinction of a species. J. R. Soc. West. Aust. 97, 297-306.

FAO, 2022. The state of world fisheries and aquaculture 2022. Towards blue transformation, The State of World Fisheries and Aquaculture (SOFIA). FAO, Rome, Italy, p. 266.

Jerry, D.R., 2001. Electrical stimulation of spermatophore extrusion in the freshwater yabby (*Cherax destructor*). Aquaculture 200(3), 317-322. https://doi.org/https://doi.org/10.1016/S0044-8486(01)00511-7.

Jones, C.M., Valverde, C., 2020. Development of mass production hatchery technology for the redclaw crayfish, *Cherax quadricarinatus*. Freshw. Crayfish 25(1), 1 - 6. https://doi.org/10.5869/fc.2020.v25-1.001.

Kowalski, R.K., Cejko, B.I., 2019. Sperm quality in fish: Determinants and affecting factors. Theriogenology 135, 94-108. https://doi.org/https://doi.org/10.1016/j.theriogenology.2019.06.009.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Rev. Aquac. 5(s1), S194-S223. https://doi.org/https://doi.org/10.1111/raq.12025.

Pérez-Cerezales, S., Martínez-Páramo, S., Beirão, J., Herráez, M.P., 2010. Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 139(6), 989. https://doi.org/10.1530/rep-10-0037.

Riesco, M.F., Valcarce, D.G., Martínez-Vázquez, J.M., Robles, V., 2019. Effect of low sperm quality on progeny: a study on zebrafish as model species. Sci. Rep. 9(1), 11192. https://doi.org/10.1038/s41598-019-47702-7.

Rigg, D., Seymour, J., Courtney, R., Jones, C., 2020. A review of juvenile redclaw crayfish *Cherax quadricarinatus* (von Martens, 1898) production in aquaculture. Freshw. Crayfish 25(1), 13-30. https://doi.org/10.5869/fc.2020.v25-1.013.

Appendix

Appendix A. Statistical summary on the effect of an un-supplemented control diet and dietary supplementation of astaxanthin (AX), cholesterol (CHO) or both AX and CHO on reproductive potential in male redclaw (*C. quadricarinatus*) broodstock between treatments at Days 0, 30, and 60. This appendix also includes the changes in reproductive potential parameters over feeding duration after acclimation for all dietary treatments combined.

Sperm viability (logit-transformed data; %)

ANOVA

Model Summary

Parameter	Sum_Squares	df	Mean_Square	F	p
Time	142.56	2	71.28	148.05	< .001
Trt	0.45	3	0.15	0.31	0.819
Residuals	71.74	149	0.48		

Anova Table (Type 2 tests)

Parameter	Coefficient	SE	95% CI	t(149)	p
(Intercept)	0.97	0.21	(0.56, 1.38)	4.70	< .001
Time (30 d)	3.35	0.20	(2.96, 3.73)	17.11	< .001
Time (60 d)	3.03	0.20	(2.64, 3.43)	15.11	< .001
Trt (CHO)	-0.03	0.16	(-0.34, 0.28)	-0.21	0.836
Trt (AX)	-0.08	0.16	(-0.39, 0.23)	-0.50	0.615
Trt (Both)	0.07	0.16	(-0.25, 0.38)	0.43	0.669

Model: logit.via ~ Time + Trt (155 Observations)
Residual standard deviation: 0.694 (df = 149)
R2: 0.668; adjusted R2: 0.657

Pairwise comparison - 30 d

Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin30 - Control30 == 0	-0.30	2.03	(-5.58, 4.97)	-0.15	0.999
Cholesterol30 - Control30 == 0	0.04	2.03	(-5.24, 5.32)	0.02	> .999
AstaChol30 - Control30 == 0	0.27	2.03	(-5.01, 5.55)	0.13	> .999
Astaxanthin30 - Cholesterol30 == 0	-0.35	2.03	(-5.62, 4.93)	-0.17	0.998
Astaxanthin30 - AstaChol30 == 0	-0.58	2.03	(-5.85, 4.70)	-0.28	0.992
Cholesterol30 - AstaChol30 == 0	-0.23	2.03	(-5.51, 5.05)	-0.11	> .999

Model Summary

Model: spmviability.prcnt ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Pairwise comparison - 60 d

Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin60 - Control60 == 0	0.10	2.34	(-5.99, 6.19)	0.04	> .999
Cholesterol60 - Control60 == 0	-0.23	2.34	(-6.33, 5.86)	-0.10	> .999
AstaChol60 - Control60 == 0	-0.08	2.34	(-6.17, 6.01)	-0.03	> .999
Astaxanthin60 - Cholesterol60 == 0	0.33	2.34	(-5.76, 6.43)	0.14	0.999
Astaxanthin60 - AstaChol60 == 0	0.18	2.34	(-5.91, 6.27)	0.08	> .999
Cholesterol60 - AstaChol60 == 0	-0.15	2.34	(-6.25, 5.94)	-0.07	> .999

Model Summary

Model: spmviability.prcnt ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Sperm DNA fragmentation (logit-transformed data; %)

ANOVA

Model Summary					
Parameter	Sum_Squares	df	Mean_Square	F	p
Time	19.55	1	19.55	24.86	< .001
Trt	0.09	3	0.03	0.04	0.990
Residuals	60.56	77	0.79		

Anova Table (Type 2 tests)

Parameter	Coefficient	SE	95% CI	t(77)	p
(Intercept)	-1.40	0.24	(-1.88, -0.91)	-5.77	< .001
Time (60 d)	1.10	0.22	(0.66, 1.55)	4.99	< .001
Trt (Cholesterol)	0.09	0.27	(-0.46, 0.64)	0.33	0.744
Trt (Astaxanthin)	0.03	0.27	(-0.52, 0.57)	0.10	0.920
Trt (Both)	0.02	0.27	(-0.52, 0.57)	0.09	0.932

Model: logit.dna ~ Time + Trt (82 Observations)
Residual standard deviation: 0.887 (df = 77)
R2: 0.247; adjusted R2: 0.208

Pairwise comparison - 60 d

Model Summary					
Parameter	Coefficient	SE	95% CI	t(77)	p
Astaxanthin60 - Control60 == 0	-5.07	6.49	(-22.13, 11.99)	-0.78	0.863
Cholesterol60 - Control60 == 0	3.57	6.49	(-13.49, 20.63)	0.55	0.946
AstaChol60 - Control60 == 0	-1.90	6.49	(-18.96, 15.16)	-0.29	0.991
Astaxanthin60 - Cholesterol60 == 0	-8.64	6.49	(-25.70, 8.42)	-1.33	0.547
Astaxanthin60 - AstaChol60 == 0	-3.17	6.49	(-20.23, 13.89)	-0.49	0.961
Cholesterol60 - AstaChol60 == 0	5.47	6.49	(-11.59, 22.53)	0.84	0.834

Model: spmdnafrag.prcnt ~ Treatment (82 Observations)
R2: 1.000
p-value adjustment method: single-step

Sperm concentration (log-transformed data; cells/ml)

ANOVA

Model Summary

Parameter	Sum_Squares	df	Mean_Square	F	p
Time	2.00	2	1.00	4.71	0.010
Trt	0.56	3	0.19	0.88	0.453
Residuals	33.13	156	0.21		

Anova Table (Type 2 tests)

Parameter	Coefficient	SE	95% CI	t(156)	p
(Intercept)	12.97	0.11	(12.75, 13.20)	114.52	< .001
Time (1 month)	0.26	0.11	(0.04, 0.48)	2.36	0.020
Time (2 months)	0.06	0.12	(-0.17, 0.28)	0.50	0.617
Trt (Cholesterol)	0.10	0.10	(-0.10, 0.30)	0.95	0.343
Trt (Astaxanthin)	8.83e-04	0.10	(-0.20, 0.20)	8.67e-03	0.993
Trt (Both)	0.13	0.10	(-0.07, 0.34)	1.31	0.191

Model: logspm.ml ~ Time + Trt (162 Observations)
Residual standard deviation: 0.461 (df = 156)
R2: 0.073; adjusted R2: 0.043

Pairwise comparison - 30 d

Model Summary

Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin30 - Control30 == 0	36500.00	87653.11	(-1.91e+05, 2.64e+05)	0.42	0.976
Cholesterol30 - Control30 == 0	75031.25	87653.11	(-1.53e+05, 3.03e+05)	0.86	0.827
AstaChol30 - Control30 == 0	7500.00	87653.11	(-2.20e+05, 2.35e+05)	0.09	> .999
Astaxanthin30 - Cholesterol30 == 0	-38531.25	87653.11	(-2.66e+05, 1.89e+05)	-0.44	0.972
Astaxanthin30 - AstaChol30 == 0	29000.00	87653.11	(-1.99e+05, 2.57e+05)	0.33	0.987
Cholesterol30 - AstaChol30 == 0	67531.25	87653.11	(-1.60e+05, 2.95e+05)	0.77	0.868

Model: spm.ml ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Pairwise comparison - 60 d

Model Summary					
Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin60 - Control60 == 0	-37666.67	1.01e+05	(-3.01e+05, 2.25e+05)	-0.37	0.982
Cholesterol60 - Control60 == 0	24666.67	1.01e+05	(-2.38e+05, 2.88e+05)	0.24	0.995
AstaChol60 - Control60 == 0	86166.67	1.01e+05	(-1.77e+05, 3.49e+05)	0.85	0.830
Astaxanthin60 - Cholesterol60 == 0	-62333.33	1.01e+05	(-3.25e+05, 2.01e+05)	-0.62	0.927
Astaxanthin60 - AstaChol60 == 0	-1.24e+05	1.01e+05	(-3.87e+05, 1.39e+05)	-1.22	0.613
Cholesterol60 - AstaChol60 == 0	-61500.00	1.01e+05	(-3.24e+05, 2.01e+05)	-0.61	0.930

Model: spm.ml ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Total potential sperm concentration (TPFSC; log-transformed data; cells/ml)

ANOVA

Model Summary					
Parameter	Sum_Squares	df	Mean_Square	F	p
Time	0.14	1	0.14	0.31	0.577
Trt	1.80	3	0.60	1.31	0.278
Residuals	35.33	77	0.46		

Anova Table (Type 2 tests)

Spermatophore weight (g)

ANOVA

Model Summary					
Parameter	Sum_Squares	df	Mean_Square	F	p
Time	2.96e-03	2	1.48e-03	12.79	< .001
Trt	1.85e-04	3	6.17e-05	0.53	0.660
Residuals	0.02	156	1.16e-04		

Anova Table (Type 2 tests)

Parameter	Coefficient	SE	95% CI	t(156)	p
(Intercept)	0.03	2.64e-03	(0.02, 0.04)	11.28	< .001
Time (30 d)	6.37e-03	2.59e-03	(1.25e-03, 0.01)	2.46	0.015
Time (60 d)	0.01	2.68e-03	(7.43e-03, 0.02)	4.75	< .001
Trt (CHO)	2.69e-03	2.38e-03	(-2.01e-03, 7.39e-03)	1.13	0.260
Trt (AX)	2.15e-04	2.38e-03	(-4.48e-03, 4.91e-03)	0.09	0.928
Trt (Both)	5.65e-04	2.38e-03	(-4.13e-03, 5.26e-03)	0.24	0.812

Model: SPW ~ Time + Trt (162 Observations)
Residual standard deviation: 0.011 (df = 156)
R2: 0.150; adjusted R2: 0.122

Pairwise comparison - 30 d

Model Summary

Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin30 - Control30 == 0	5.00e-05	3.41e-03	(-8.82e-03, 8.92e-03)	0.01	> .999
Cholesterol30 - Control30 == 0	3.00e-03	3.41e-03	(-5.87e-03, 0.01)	0.88	0.816
AstaChol30 - Control30 == 0	1.00e-04	3.41e-03	(-8.77e-03, 8.97e-03)	0.03	> .999
Astaxanthin30 - Cholesterol30 == 0	-2.95e-03	3.41e-03	(-0.01, 5.92e-03)	-0.86	0.823
Astaxanthin30 - AstaChol30 == 0	-5.00e-05	3.41e-03	(-8.92e-03, 8.82e-03)	-0.01	> .999
Cholesterol30 - AstaChol30 == 0	2.90e-03	3.41e-03	(-5.97e-03, 0.01)	0.85	0.831

Model: SPW ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Pairwise comparison - 60 d

Model Summary

Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin60 - Control60 == 0	-1.33e-04	3.94e-03	(-0.01, 0.01)	-0.03	> .999
Cholesterol60 - Control60 == 0	4.93e-03	3.94e-03	(-5.31e-03, 0.02)	1.25	0.595
AstaChol60 - Control60 == 0	1.60e-03	3.94e-03	(-8.65e-03, 0.01)	0.41	0.977
Astaxanthin60 - Cholesterol60 == 0	-5.07e-03	3.94e-03	(-0.02, 5.18e-03)	-1.29	0.574
Astaxanthin60 - AstaChol60 == 0	-1.73e-03	3.94e-03	(-0.01, 8.51e-03)	-0.44	0.971
Cholesterol60 - AstaChol60 == 0	3.33e-03	3.94e-03	(-6.91e-03, 0.01)	0.85	0.833

Model: SPW ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Gonadosomatic index (log-transformed data; GSI)

ANOVA

Model Summary

Parameter	Sum_Squares	df	Mean_Square	F	p
Time	0.64	2	0.32	7.04	0.001
Trt	0.06	3	0.02	0.44	0.727
Time:Trt	0.27	6	0.05	1.00	0.428
Residuals	6.54	144	0.05		

Anova Table (Type 2 tests)

Parameter	Coefficient	SE	95% CI	t(150)	p
(Intercept)	-0.60	0.05	(-0.70, -0.49)	-11.29	< .001
Time (30 d)	0.17	0.05	(0.06, 0.27)	3.20	0.002
Time (60 d)	0.20	0.05	(0.09, 0.31)	3.71	< .001
Trt (CHO)	0.04	0.05	(-0.06, 0.13)	0.76	0.449
Trt (AX)	0.05	0.05	(-0.04, 0.15)	1.11	0.267
Trt (Both)	0.03	0.05	(-0.06, 0.12)	0.63	0.529

Model: log.gsi ~ Time + Trt (156 Observations)
Residual standard deviation: 0.213 (df = 150)
R2: 0.096; adjusted R2: 0.066

Pairwise comparison - 30 d

Model Summary

Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin30 - Control30 == 0	0.15	0.06	(2.09e-04, 0.30)	2.60	0.050
Cholesterol30 - Control30 == 0	0.16	0.06	(8.06e-03, 0.31)	2.73	0.035
AstaChol30 - Control30 == 0	0.08	0.06	(-0.07, 0.23)	1.40	0.500
Astaxanthin30 - Cholesterol30 == 0	-7.85e-03	0.06	(-0.16, 0.14)	-0.14	> .999
Astaxanthin30 - AstaChol30 == 0	0.07	0.06	(-0.08, 0.22)	1.20	0.630
Cholesterol30 - AstaChol30 == 0	0.08	0.06	(-0.07, 0.23)	1.33	0.544

Model: GSI ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Pairwise comparison - 60

Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin60 - Control60 == 0	-0.03	0.07	(-0.21, 0.14)	-0.52	0.954
Cholesterol60 - Control60 == 0	-9.93e-03	0.07	(-0.18, 0.16)	-0.15	0.999
AstaChol60 - Control60 == 0	-0.06	0.07	(-0.24, 0.11)	-0.92	0.793
Astaxanthin60 - Cholesterol60 == 0	-0.03	0.07	(-0.20, 0.15)	-0.37	0.982
Astaxanthin60 - AstaChol60 == 0	0.03	0.07	(-0.15, 0.20)	0.40	0.978
Cholesterol60 - AstaChol60 == 0	0.05	0.07	(-0.12, 0.23)	0.77	0.866

Model Summary

Model: GSI ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Hepatosomatic index (HSI)

ANOVA

Parameter	Sum_Squares	df	Mean_Square	F	p
Time	7.61	2	3.80	6.34	0.002
Trt	0.23	3	0.08	0.13	0.944
Residuals	93.03	155	0.60		

Model Summary

Anova Table (Type 2 tests)

Parameter	Coefficient	SE	95% CI	t(155)	p
(Intercept)	5.15	0.19	(4.77, 5.53)	26.97	< .001
Time (1 mo)	0.50	0.19	(0.13, 0.87)	2.65	0.009
Time (2 mos)	0.69	0.19	(0.31, 1.07)	3.56	< .001
Trt (Cholesterol)	-0.03	0.17	(-0.37, 0.31)	-0.19	0.852
Trt (Astaxanthin)	2.45e-03	0.17	(-0.34, 0.34)	0.01	0.989
Trt (Both)	-0.09	0.17	(-0.43, 0.25)	-0.53	0.599

Model: HIS ~ Time + Trt (161 Observations)
Residual standard deviation: 0.775 (df = 155)
R2: 0.077; adjusted R2: 0.048

Pairwise comparison - 30 d

Model Summary					
Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin30 - Control30 == 0	-0.59	0.32	(-1.43, 0.25)	-1.82	0.266
Cholesterol30 - Control30 == 0	-0.35	0.32	(-1.19, 0.48)	-1.10	0.690
AstaChol30 - Control30 == 0	-0.52	0.32	(-1.36, 0.32)	-1.62	0.371
Astaxanthin30 - Cholesterol30 == 0	-0.23	0.32	(-1.07, 0.60)	-0.72	0.887
Astaxanthin30 - AstaChol30 == 0	-0.07	0.32	(-0.90, 0.77)	-0.20	0.997
Cholesterol30 - AstaChol30 == 0	0.17	0.32	(-0.67, 1.01)	0.52	0.954

Model: HIS ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step

Pairwise comparison - 60 d

Model Summary					
Parameter	Coefficient	SE	95% CI	t(153)	p
Astaxanthin60 - Control60 == 0	0.12	0.37	(-0.85, 1.08)	0.31	0.989
Cholesterol60 - Control60 == 0	-0.14	0.37	(-1.11, 0.83)	-0.37	0.982
AstaChol60 - Control60 == 0	-0.15	0.37	(-1.12, 0.82)	-0.40	0.978
Astaxanthin60 - Cholesterol60 == 0	0.26	0.37	(-0.71, 1.22)	0.69	0.902
Astaxanthin60 - AstaChol60 == 0	0.27	0.37	(-0.70, 1.23)	0.72	0.891
Cholesterol60 - AstaChol60 == 0	0.01	0.37	(-0.96, 0.98)	0.03	> .999

Model: HIS ~ Treatment (162 Observations)
R2: 1.000
p-value adjustment method: single-step