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

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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 depends 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 optimized conventional and advanced functional tools. Sexually mature male redclaw ($n = 33$) were collected from Ross River Dam in 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 fertilization-spike structure. The mean ± SEM sperm viability, DNA fragmentation, concentration, and TPFSC were 65.2 ± 3.9 %, 17.2 ± 2.5 %, 42.5 ± 5.1 × 10^4 cells/mL, and 23.6 ± 3.4 × 10^4 cells/mL, respectively.

Spermatophore weight showed a positive association with sperm concentration ($p = 0.001$) and total potential fertile sperm concentration (TPFSC; $p < 0.001$), and a negative association with sperm DNA fragmentation ($p = 0.022$). Conversely, sperm viability showed no significant relationship with spermatophore weight ($p = 0.188$) but was 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 insight into the relationships between sperm quality parameters and body or spermatophore weights. Our approach may inform the selection of fertile male broodstock and enhanced strategies for sperm harvesting in order to facilitate selective breeding programs for commercial redclaw aquaculture.

1. Introduction

Redclaw crayfish (*Cherax quadricarinatus*) are increasingly recognised for their farming and intensification potential in aquaculture (Rigg et al., 2020; Haubrock et al., 2021). They are non-burrowing, large, hardy, and fast-growing freshwater crayfish native to northern Australia (Jones, 2011; Haubrock et al., 2021). 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, 2000; Haubrock et al., 2021). In 2023, global production of redclaw crayfish reached 650 t, equating to US$ 6.6 million (FAO, 2024), a volume that is projected to continue to expand (Irvin et al., 2018). Despite its economic potential, the redclaw farming industry faces multiple challenges, including an insufficient seedstock supply, a

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limited production season, and economically inefficient and small-scale production (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 survival rates (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 (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 (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 (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.

While male fertility is ultimately measured by fertilization and hatching rates, these methods are time-consuming, influenced by egg quality, and can yield variable results (Adams et al., 2003). 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). The reproductive efficiency of farmed crustaceans has primarily focused on egg and embryo, rather than sperm quality. However, the competence of eggs and spermatozoa affects fertilization success, embryonic and larval development, and survival (Bobe and Labbé, 2010; Martín-Manzo et al., 2024). As such, the assessment of sperm quality should be a key consideration when evaluating and optimizing reproductive productivity within hatcheries (Rurangwa et al., 2004; Marc et al., 2021). Various techniques have been used to assess sperm quality in aquaculture-relevant decapods, such as shrimp (Duangjai et al., 2023), freshwater prawns (Poljaroen et al., 2010), and spiny lobsters (Fatihah et al., 2016), including standard evaluations of concentration, morphology, and viability, as well as advanced methods such as DNA integrity analysis (Erraud et al., 2019; Aquino et al., 2022).

In all species, subfertile males can limit the production of offspring (Rurangwa et al., 2004; Riesco et al., 2019). For example, while the degree of sperm DNA damage in mammals does not consistently predict fertilization rates (Kumar et al., 2013), it is linked to increased apoptosis and cellular damage within embryos, leading to delayed or arrested embryonic development, reduced blastocyst formation and pregnancy rates, and higher rates of abortion (Simon et al., 2014; Peña Jr. et al., 2017; Peña Jr. et al., 2019; Zheng et al., 2018). 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).

In decapod aquaculture, the method used for sperm collection plays a critical role in preserving sperm integrity and influencing the accuracy of downstream reproductive assessments. Different techniques, including dissection, manual extrusion, and electroejaculation, have been applied across species with varying degrees of success. In *Penaeus setiferus*, for instance, electroejaculation has been associated with reduced live sperm counts, increased abnormalities, and tissue necrosis within ampullae, often accompanied by bacterial contamination (Rosas et al., 1993). While dissection yields high-quality samples, it is inherently terminal and therefore unsuitable for broodstock conservation and reproductive planning. Manual extrusion, commonly performed in penaeid shrimp by pressing the coxae of the fifth walking legs (Beirão et al., 2019; Diggles, 2019), can induce genital inflammation and melanisation, particularly when done too frequently or improperly (Braga et al., 2018). These outcomes highlight how the choice and handling of sperm collection techniques can introduce artifacts or damage, thereby compromising sperm structure, viability, and overall function. Given the rigid exoskeleton of redclaw crayfish, electroejaculation offers a non-lethal, practical, and species-appropriate method for spermatophore collection. It enables consistent sperm retrieval under controlled conditions, ideal for high-resolution sperm quality assessment and the development of diagnostic tools for broodstock selection and reproductive management.

Traditional sperm quality assessments, including evaluations of motility, morphology, and concentration, have long served as indicators of fertility. However, their predictive value can be context-dependent and may not always reliably reflect fertilization potential, particularly in non-competitive or controlled mating scenarios (Gillan et al., 2005; Duangjai et al., 2023). For instance, sperm concentration has been strongly associated with paternity success in species exhibiting sperm competition or promiscuous mating systems, such as certain fish and mammals; however, it alone does not account for larval quality outcomes (Fitzpatrick, 2020; Marc et al., 2024). These traditional assays do not always correlate with fertilizing capacity or early embryonic development, making them inadequate on their own for assessing fertility (Graham et al., 1990; Gillan et al., 2005; Cabrita et al., 2014; Duangjai et al., 2023). In addition, these methods are not directly applicable to redclaw crayfish, as their spermatozoa are non-motile and lack flagella (Beach and Talbot, 1987; Kouba et al., 2015; Subramoniam, 2017a, 2017b), making motility, a key biomarker for fertilization in other species, unsuitable for this decapod (Lezcano et al., 2004; Lewis and Ford, 2012). Optical microscopy is commonly used to evaluate sperm morphology in crustaceans; however, its application is often limited by the absence of conventional structural features such as flagella, mitochondria, or acrosomes (Lezcano et al., 2004; Gillan et al., 2005; Braga et al., 2013; Aquino et al., 2022). This lack of distinct morphological landmarks renders assessments more subjective and potentially inconsistent. Assessing a single sperm quality biomarker is insufficient for robust reproductive data; hence, combining multiple traits provides a more accurate predictor of male fertility and fertilizing capacity (Graham et al., 1980, 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 coupled with fluorescent microscopy or flow cytometry to detect the integrity of DNA as well as plasma, acrosome, and mitochondrial membranes is increasingly applied to evaluate sperm quality in aquaculture species (De Baulny et al., 1997; Gillan et al., 2005; Liu et al., 2007; Favret and Lynn, 2010; Qiu et al., 2011; Marc et al., 2021, 2024), and specifically decapods (Lezcano et al., 2004; Lewis and Ford, 2012; Erraud et al., 2018; Aquino et al., 2022; Duangjai et al., 2023). 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).

Declining fertility across successive generations of captive broodstock has been well-documented in aquaculture species, often due to domestication effects, inbreeding, environmental stress, or suboptimal rearing conditions (Leung-Trujillo and Lawrence, 1987; Rendón Rodríguez et al., 2007; Silva et al., 2015; Gilroy and Litvak, 2019; Sheikh et al., 2019). Consequently, wild-caught individuals remain the gold standard for evaluating reproductive performance, providing a critical reference point for assessing and optimizing broodstock management strategies in hatchery settings.

Despite the availability of studies describing sperm morphology (Beach and Talbot, 1987; An et al., 2011; Kouba et al., 2015) and estimating sperm counts in redclaw crayfish (Bugnot and López Greco, 2009), a comprehensive profile encompassing multiple sperm quality traits remains to be determined. This limits the ability of aquaculture facilities to screen and select for highly fertile male broodstock. Thus, this study aimed to combine traditional techniques with more advanced fluorescent and flow cytometric methods to describe sperm morphology and quantify sperm concentration, viability and DNA integrity as predictors of male fertility in wild-caught redclaw crayfish.

2. Materials and methods

2.1. 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 and Lusiastuti, 2015). Animals were weighed and measured before being subjected to electroejaculation. Crustaceans are exempt from animal ethics requirements (JCU Animal Ethics Committee); however, this study still conforms to ACH protocols for ethical animal treatment and reflects industry's best practices.

2.2. 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 intermolt stage were measured after being anaesthetized in chilled freshwater (15 °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 V, 0.01 A, and cycle frequency between 50 and 60 Hz with 1–2 s 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 (370.1 mM NaCl, 15.0 mM KCl, 8.6 mM H_3BO_3, 4.8 mM NaOH, and 20.0 mM $MgSO_4 \bullet 7H_2O$ at pH 7.4) immediately after extrusion and weighed (Leung-Trujillo and Lawrence, 1987). The use of CFS solution is a recommended medium for sperm quality assessments and is widely used for other decapod crustaceans since the absence of calcium prevents initiation of the acrosome reaction in spermatozoa (Samuel et al., 1999; Vuthiphandchai et al., 2007; Xu et al., 2014; Feng et al., 2018). Melanised spermatophores were not observed in the current study.

To characterize 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 at room temperature (RT, 22–24 °C) and protected from light. 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 decision to examine and photograph spermatophores at 24 h post-electroejaculation was guided by both biological and practical considerations, as this timepoint coincided with the onset of hydration described by López Greco and Lo Nostro (2008). The second tube was examined 96 h post-electroejaculation, the earliest time point at which spermatozoa are naturally released from spermatophores without physical intervention (López Greco and Lo Nostro, 2008). Observations and imaging were performed using a stereomicroscope (Olympus SZX7-ILLT, Tokyo, Japan) equipped with a Basler ace acA2440-75uc camera (Ahrensburg, Germany), and a phase-contrast microscope (Olympus BX53, Tokyo, Japan) coupled with a Basler Aviator avA1000-gc camera (Ahrensburg, Germany).

To analyze 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 maximized by 10 x gentle repeat-aspiration using a 1000 μL pipette tip, 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.

2.3. Sperm number, concentration, and morphology

The concentration and total number of spermatozoa in each spermatophore were manually calculated 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 thoroughly cleaned 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 following World Health Organisation guidelines (WHO, 2021) and examined in its hydrated state under phase-contrast microscopy 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 1.53 t) 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 the baseline range of normal morphology, 300 spermatozoa from 20 randomly selected male redclaw were assessed. The formulae below were used to calculate key sperm morphology parameters (Marc et al., 2021).

$$\text{Head ratio (ellipticity)} = L/W$$

$$\text{Head area} = \pi^{*} L/2^{*} W/2$$

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

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

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

2.4. 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 optimized 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^+) were 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 % 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 xg, 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 °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 permeabilization protocol disrupted the plasma membrane in 93.3 ± 0.3 % (mean ± SEM) of spermatozoa.

Identical conditions were used for H_{342} and PI staining of test samples; however, staining was conducted 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 nuclei of membrane-disrupted (dead) spermatozoa were stained pink/red by PI. Double-stained H^+_{342}/PI^+ spermatozoa were considered damaged/dead cells. Flow cytometry was performed two hours after generating the single-cell suspension, and the percentage of viable spermatozoa was determined for each test sample (see Supplementary Figs. S1 and S2 for details about staining specificity and gating strategy used in flow cytometry, respectively).

2.5. 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 Jr. 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 without and with H_{342} staining (U_1 & U_2, respectively), negative controls in label solution only (without terminal transferase) without and with H_{342} stain (N_1 & N_2, respectively), and *DNase*-treated FITC-positive controls without and with H_{342} stain (P_1 & P_2, respectively; (Peña Jr. et al., 2019; Marc et al., 2021). Controls and test samples were fixed one hour after generating a single-cell suspension and on the same day as sperm viability testing. Fixation was performed with constant agitation (450 rpm) using freshly prepared 4 % paraformaldehyde in CFS solution (pH 7.4) for 1 h at RT. Samples were centrifuged (200 xg, 5 min), and sperm pellets were washed twice with CFS solution. Sperm cells were permeabilized with 0.1 % Triton X-100 in 0.1 % sodium citrate (2 min, 4 °C), centrifuged (200 xg, 5 min), and then re-suspended in 200 μl CFS solution and stored at 4 °C overnight.

Fixed and permeabilized positive controls (P_1 & P_2) were established by incubating samples with 10 U/μL of recombinant *DNase* 1 dissolved in Roche Buffer 2 (RB_2), containing 10 mM Tris-HCl, 10 mM NaCl, 5 mM $MnCl_2 \bullet 4H_2O$, 0.1 mM $CaCl_2$, 25 mM KCl (0.10 U/μL final concentration of *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, prior to flow cytometry to assess the percentage of DNA-damaged spermatozoa among the total number of identified spermatozoa within the gated area (see Supplementary Figs. S1 and S3 for details on the staining specificity and flow cytometry gating strategy, respectively).

2.6. Flow cytometry

To quantify viability and DNA fragmentation, 1.0×10^5 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. Instrument performance was standardized 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. Positive and negative controls were used to define gating regions and single colour controls for spectral overlap compensation for H_{342}/PI (sperm viability) and H_{342}/TUNEL-FITC (DNA fragmentation). 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 (see Supplementary Figs. S2 and S3 for details on flow cytometry gating strategy used to assess sperm viability and DNA fragmentation, respectively).

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

Total Potential Fertile Sperm Concentration (TPFSC) is a novel

metric introduced in this study to assess the concentration of sperm with fertilizing potential in *C. quadricarinatus*. Given that redclaw crayfish spermatozoa are non-motile and lack flagella, sperm motility was not considered in this study. Instead, we developed the TPFSC formula to incorporate sperm concentration, DNA fragmentation, and viability as key factors for evaluating sperm quality.

The formula for TPFSC is as follows:

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

where,

SC = sperm concentration (cells/mL).
DF = DNA fragmented spermatozoa (%).
VS = viable spermatozoa (%).

2.8. 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 body or spermatophore weight 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 summarized 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), only the relationship between male redclaw body weight and sperm quality was further assessed. Diagnostic plots were employed to detect heterogeneity of variance and non-normal distribution of data residuals. Logarithmic transformation was applied to address non-normality and heteroscedasticity in the sperm concentration, count, and TPFSC data prior to regression analysis, while logit transformation was employed for sperm viability and DNA fragmentation, as these data are proportions ranging between 0 and 1 (Seiffert et al., 2024). The data transformations were performed to stabilize variance and improve the distribution of residuals, ensuring a more normal distribution. These adjustments were essential to meet the assumptions of the regression model, thereby enhancing the accuracy and reliability of the statistical inference. The raw data were used to plot regression graphs between body or spermatophore weights and sperm quality indicators, revealing significant relationships. Data variables were summarized as mean ± SEM to reflect the precision of the sample mean and the reliability of the estimates.

3. Results

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 (Fig. 1 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 (Fig. 1 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 (Fig. 1 c – d).

Redclaw spermatozoa were immotile, adhesive, 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 medio-posterior aspect of the cell (Supplementary Fig. S1 b & Fig. 2 b - yellow arrows), (ii) an acrosome-like cap (Supplementary Fig. S1 b - asterisk,), and the presence of a putative fertilization-spike structure in many, but not all spermatozoa (white arrows in Fig. 2 a – b). Based on examination of 2000 spermatozoa from 10 randomly selected males, 19.6 ± 1.2 % (mean ± SEM) of the redclaw sperm population were identified as having this presumptive fertilization-spike structure. Spermatozoa with the fertilization-spike structure had a head length of 13.7 ± 0.5 µm, a head width of 11.7 ± 0.5 µm, and a spike length of 47.4 ± 3.3 µm (mean ± SEM; n = 100 spermatozoa). Sperm parameters,

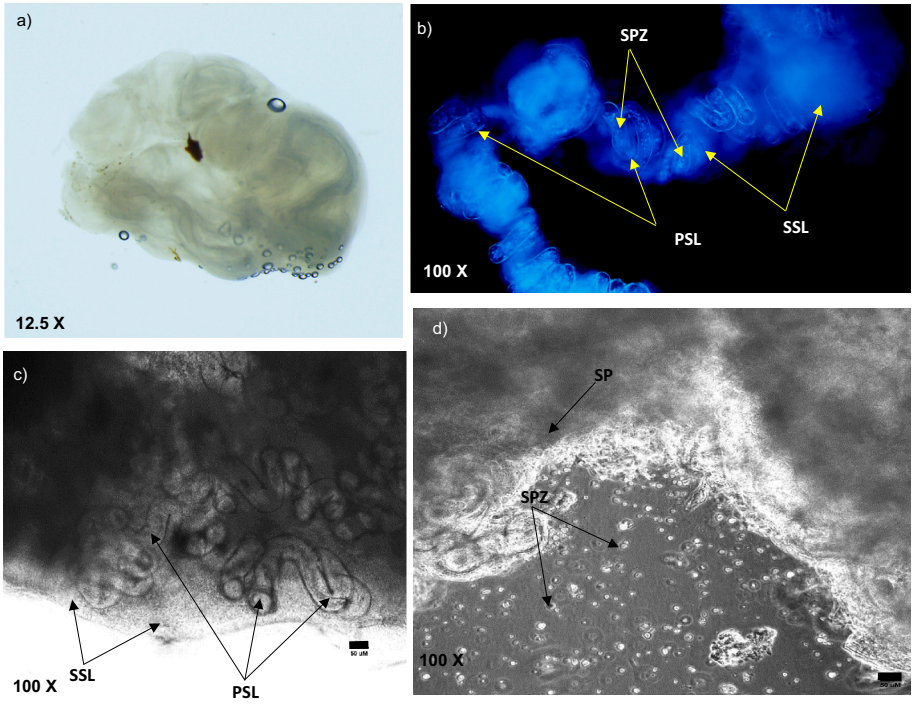

Fig. 1. Sub-structure of the extruded spermatophore of *C. quadricarinatus*. (a) Intact spermatophore 10 min after extrusion (stereo microscopy); (b) H_{342}-stained spermatophore showing distinct primary and secondary layers 24 h after extrusion (inverted fluorescent microscopy); c) spermatophore showing 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.

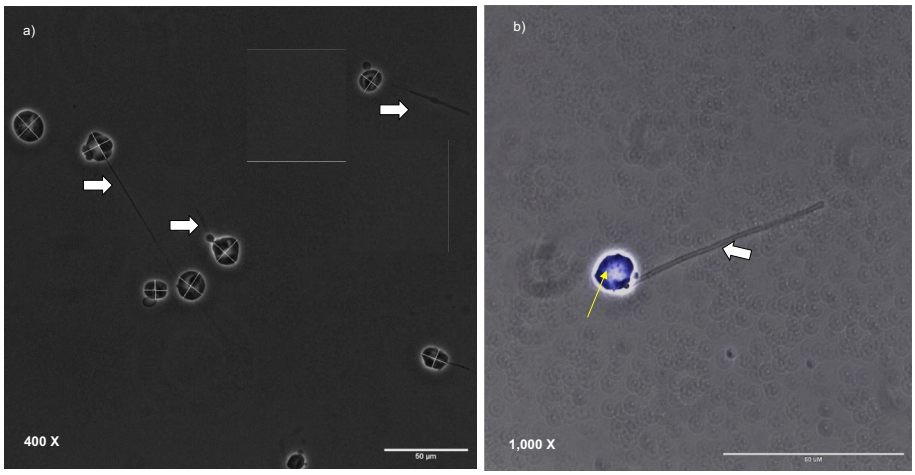

Fig. 2. Phase contrast micrographs of *C. quadricarinatus* spermatozoa. (a) Measurement of the sperm head (length and width) and presence of a putative fertilization-spike structure (white arrows) (b) Hoechst 33342 stained nuclear area (yellow arrow) of spermatozoa and putative fertilization-spike structure (white arrow). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

including head length, width, area, ratio, perimeter, surface area, and volume, are shown in Table 1. Head length, width and ratio were consistent and confirm a mildly elliptical shape. However, calculations used to derive several other parameters, including head area, surface area, and volume, appeared to amplify the variability between individual spermatozoa, which may be attributed to the inherent biological variability of the samples.

Flow cytometry was successfully able to quantify sperm subpopulations based on viability and DNA integrity. In the H_{342}/PI scatter plot (Fig. 3 a), a clear distinction was observed between viable and non-viable spermatozoa, with most events clustered in the viable quadrant (Hoechst 33342-positive, PI-negative). Conversely, in the TUNEL-FITC/H_{342} scatter plot (Fig. 3 b), the proportions of DNA-intact to DNA-damaged spermatozoa were similar in this particular male. The mean (± SEM) values of different sperm quality parameters for wild-caught adult redclaw are shown in Table 2. While mean values were generally consistent, the range of several parameters (sperm concentration, sperm count, DNA damage and TPFSC) was quite broad 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). Sperm viability decreased by 0.47 % for every gram increase in the body weight of male redclaw crayfish (Fig. 4 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). Sperm concentration and TPFSC increased by 17,404 and 11,226 cells/mL, respectively, for every milligram increase in spermatophore weight (Fig. 4 b – c). However, sperm DNA fragmentation decreased by 0.73 % for every milligram increase in spermatophore weight (Fig. 4 d).

Sperm concentration was moderately and negatively correlated with DNA fragmentation ($p = 0.008$; Table 3). Sperm DNA fragmentation decreased by 2 % on average for every 10,000 cells/mL increase in sperm concentration (Fig. 4 e). No direct association was found between sperm viability, concentration, count, or DNA fragmentation in wild-caught male redclaw spermatozoa (Table 3).

Table 1
Characterization 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)	1786.3 ± 79.1	1.1–8630.8
Head volume (μm^3)	2495.9 ± 177.0	57.4–21,130.9

4. Discussion

Using a combination of conventional and advanced functional sperm quality diagnostic tools, we describe, for the first time, sperm quality data for wild-caught male redclaw crayfish (*C. quadricarinatus*) from North Queensland, Australia. A putative fertilization-spike 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, moderate sperm viability, and broadly variable sperm quality among males. Additionally, heavier animals had lower sperm viability, while heavier spermatophores had higher sperm concentration, less DNA damage, and improved total potential fertile sperm concentration (TPFSC). The broad range observed in this study among males across different sperm quality parameters, highlights the potential of these tools to screen male broodstock to select highly fecund individuals, thereby improving commercial aquaculture systems.

The initial step to evaluate sperm quality in decapod crustaceans involves spermatophore extraction (Beirão et al., 2019; Aquino et al., 2022). This traditionally involves dissection of the vas deferens, a lethal sampling procedure that requires sacrifice of male broodstock, thus precluding their future participation in breeding programs (Bugnot and López Greco, 2009). While non-lethal alternatives such as manual stripping and electroejaculation exist (Bart et al., 2006; Jerry, 2001), manual stripping is often ineffective or inconsistent in hard-shelled species like redclaw crayfish due to their rigid exoskeleton. Moreover, in *penaeids*, manual stripping has been linked to gonophore melanisation and increased mortality (Chamberlain et al., 2009; Alfaro-Montoya, 2010). Electroejaculation, by contrast, offers a reliable, non-lethal method for spermatophore collection, preserving male viability and reproductive potential. This technique has been successfully applied in several freshwater crayfish species, including *Cherax* species (Jerry,

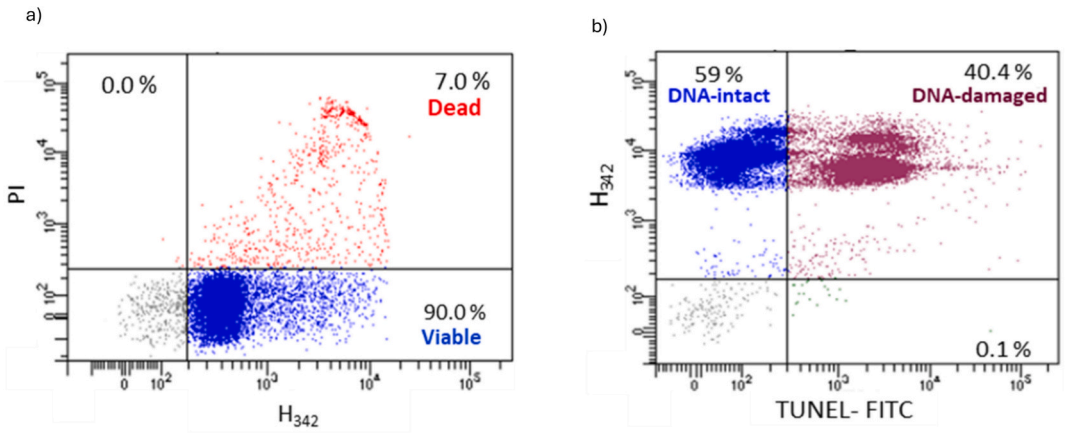

Fig. 3. Assessment of viability and DNA fragmentation in subpopulations of *C. quadricarinatus* spermatozoa. (a) A test sample showing subpopulations of viable (propidium iodide-negative) and dead (propidium iodide-positive) spermatozoa, assessed using Hoechst 33342/propidium iodide (H_{342}/PI) staining and flow cytometry, presented on an H_{342}/PI scatter plot. (b) A test sample displaying subpopulations of intact (FITC-negative) and DNA-damaged (FITC-positive) spermatozoa, evaluated using the Terminal deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) assay and flow cytometry, shown on an H_{342}/TUNEL-FITC scatter plot. FITC, fluorescein isothiocyanate; H_{342}, Hoechst 33342; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

Table 2
Mean (± SEM) values of different sperm quality parameters for wild-caught adult redclaw crayfish (*C. quadricarinatus*).

Parameter	mean ± SEM (n = 33 animals)	Range (min-max)
SP 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

SP - spermatophore; TPFSC - total potential fertile sperm concentration.

2001; Kouba et al., 2015), and involves using a mild electrical stimulus to induce muscle contraction and release of the spermatophore (Diggles, 2019). Establishing a standardized electroejaculation protocol for redclaw hatcheries holds significant potential for advancing reproductive technologies such as artificial fertilization and sperm cryopreservation (Aquino et al., 2022).

Electroejaculation, followed by spermatophore suspension in CFS solution, was a reliable and non-lethal method for extruding and processing 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 it to release spermatozoa to fertilize eggs (Jones et al., 1996; López Greco and Lo Nostro, 2008).

In crustaceans, single-sperm suspensions have been achieved using mechanical disruption of the spermatophore after collection. This involves either maceration and repeat pipetting in a semen extender solution to disrupt spermatophore walls, or gentle homogenisation using a tissue grinder (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 °C 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 sperm aggregates and

Table 3
Relationship between body weight, spermatophore weight and sperm quality parameters in wild-caught adult male 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
	SP weight (g)	0.05	0.197		No
SP 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 (−)**
	TPFSC (log-transformed data; TPFSC/ml)	**0.33**	**< 0.001**	**0.58**	**Moderate (+)**
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

SP - spermatophore; TPFSC - total potential fertile sperm concentration.

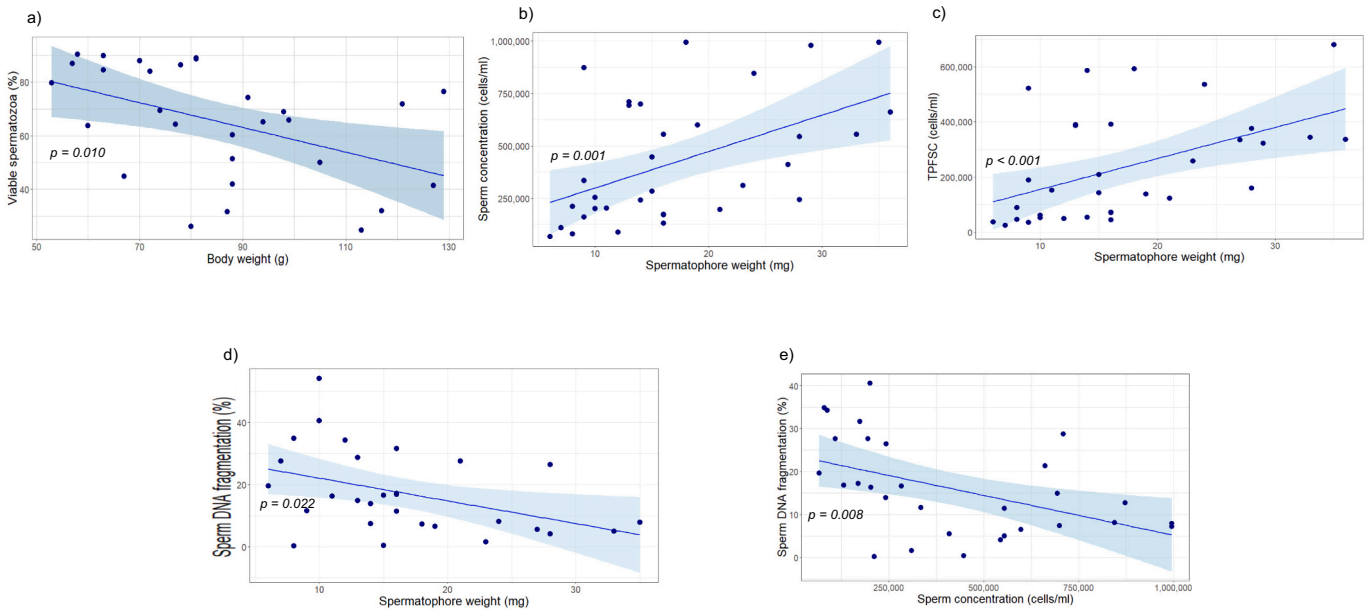

Fig. 4. Linear regressions with a 95 % confidence interval (blue band) between (a) body weight and sperm viability, (b) spermatophore weight and sperm concentration, (c) spermatophore weight and total potential fertile sperm concentration, (d) spermatophore weight and sperm DNA fragmentation, and (e) sperm concentration and DNA fragmentation. All regressions are significant at $p \leq 0.05$. TPFSC, total potential fertile sperm concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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.

In this study, Hoechst 33342 (H_{342}), a cell-permeable, DNA-specific fluorescent dye, was used to evaluate the permeability of redclaw crayfish spermatophores and facilitate nuclear staining. At 24 h post-extrusion, successful staining confirmed that the outer layers of the spermatophore became sufficiently penetrable, allowing the dye to access and bind to DNA within the sperm nuclear membrane. This time-dependent increase in permeability was critical for the adequate preparation of a single-cell sperm suspension, which was achieved by manually sectioning the spermatophore and gently releasing the spermatozoa via pipetting. In contrast, spermatophores stained at earlier timepoints (e.g., 30–60 min post-collection) remained too compact and impermeable, resulting in no observable staining and suggesting limited dye penetration shortly after extrusion. Preliminary extraction trials also revealed that within 30–60 min post-electroejaculation, the spermatophores retained a firm, rigid consistency that hindered efficient sperm recovery. Although extraction within 10 min was occasionally possible, the material was highly viscous and sticky, leading to contamination with cellular debris and necessitating additional purification steps. By 24 h post-extrusion, the spermatophore matrix appeared to undergo partial hydration, consistent with the progressive softening previously described by López Greco and Lo Nostro (2008). This hydration likely contributed to the improved recovery of intact spermatozoa using gentle pipetting techniques. At this stage, cleaner single-cell suspensions were obtained with minimal debris, providing an optimal balance between ease of extraction and enhanced sample quality.

In this study, electroejaculation yielded an average of 0.02 ± 0.001 g of spermatophore with an estimated sperm count of ~3.0×10^7 cells per gram. Complementary findings from our separate study showed that a 1-cm segment of the distal vas deferens (DVD) collected by lethal post-mortem dissection, weighed 0.06 ± 0.005 g and contained ~2.0×10^7 spermatozoa (Aquino et al., 2022, unpublished). These results align with the range reported by Bugnot and López Greco (2009), who documented 10^7–10^9 spermatozoa per 1-cm DVD segment in redclaw crayfish. While methodological differences exist, our study estimates sperm count per gram from electroejaculated samples, and Bugnot and López Greco (2009) quantify cells per DVD segment, with the consistency in sperm density supporting the reliability of both approaches. These findings reinforce the utility of electroejaculation as a non-lethal, practical technique for collecting spermatophores with adequate yield for reproductive assessment in redclaw crayfish. Further refinement of spermatophore handling and dissolution protocols, including optimization of holding media, is recommended to maximise sperm recovery and assess the influence of extraction methods on sperm quality.

Abnormal sperm morphology in decapod crustaceans is often used as an indicator of subfertility or atypical spermatogenesis (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 assessment of sperm morphology and quality in wild-caught freshwater redclaw crayfish. The present study provides the first two-dimensional morphometric data for redclaw crayfish spermatozoa, reporting an average head length of 16.3 ± 0.3 μm and width of 14.2 ± 0.3 μm, indicating a slightly elliptical morphology similar to that described by An et al. (2011). Previous reports describe redclaw sperm as irregular in shape, immotile, and lacking spikes or flagella (Beach and Talbot, 1987; Kouba et al., 2015). In contrast, the present study identified three morphological features: an elliptical sperm head containing a decondensed nucleus, an acrosome-like cap, and, occasionally, a putative fertilization-spike structure (Fig. 2). While limited comparative data exist, marine shrimp *Pandalopsis japonica*, possesses a larger, cup-shaped sperm head measuring 52–55 μm in length and 24–26 μm in width (Kim et al., 2003). Most studies of decapod sperm morphology report a single measurement of sperm head diameter, including 8–9 μm in the giant freshwater prawn, *Macrobrachium rosenbergii* (Poljaroen et al., 2010), 2–3 μm in banana shrimp, *Penaeus merguiensis* (Memon et al., 2012), and 5–6 μm in black tiger shrimp *Penaeus monodon* (Gomes and Honculada-Primavera, 1993). Defining baseline normal morphometric characteristics for redclaw may serve as a practical screening criterion to select high-performing male broodstock in aquaculture

settings.

The detection of a presumptive fertilization-spike structure in redclaw crayfish spermatozoa in this study may be attributed to the gentler sperm extraction and visualisation methods employed, which likely preserved fragile structural features. Unlike previous studies that assessed crayfish sperm morphology using fixed and dehydrated specimens through histological preparation, 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), this study utilised phase-contrast microscopy on fresh, unfixed samples. Techniques involving fixation and dehydration of spermatozoa may cause cell shrinkage or structural distortion, potentially obscuring or detaching delicate appendages such as fertilization-spike projections (Cabrita et al., 2009).

Post-mating spermatophore storage in decapods is associated with structural changes that prepare spermatozoa for fertilization (Subramoniam, 2017c; Farhadi and Harlıoğlu, 2019). These changes resemble mammalian capacitation, during which sperm gain the ability to undergo the acrosome reaction. While previous studies on *Cherax* species within the genus *Parastacidae* have not reported the presence of true flagella or radial arms (Beach and Talbot, 1987; An et al., 2011; Kouba et al., 2015), acrosomal horns or spike-like projections, which are potentially involved in sperm maturation and fertilization, have been documented in other freshwater crayfish genera such as *Astacidae* and *Cambaridae* (Niksirat et al., 2013; Niksirat et al., 2014). In the present study, a putative fertilization-spike was observed in redclaw spermatozoa as a filamentous projection under phase-contrast microscopy, differing in appearance from the rigid spine-like structures previously described in other decapod species with unistellate spermatozoa (Poljaroen et al., 2010). A similar filament has been described in the lobster *Homarus americanus*. During sperm evagination, the acrosome turns inside out, and the nucleus and spikes move into the former acrosomal space. A leading filament then forms and guides the sperm toward the egg, and together with released enzymes assists penetration of the thick chorion (Talbot and Chanmanon, 1980; Subramoniam, 2017c). Comparable acrosomal reactions have also been reported in crabs such as *Cancer setosus* (Dupré et al., 2012) and *Eriocheir sinensis* (Wang et al., 2015), where a distinct filament is projected as part of the fertilization process.

One plausible explanation for the inconsistent presence of the presumptive fertilization-spike structure among redclaw spermatozoa is the asynchronous nature of spermatogenesis across different seminal acini in a spermatophore. As noted by An et al. (2011), while germ cell development within a single acinus is typically synchronous, it can vary considerably between acini. As a result, spermatozoa within a single spermatophore may be at different maturational stages, and not all may exhibit accessory structures such as the fertilization-spike at the same time. This structure may serve a functional role in fertilization, potentially enhancing sperm-egg interaction or aiding adhesion during external fertilization, particularly as its presence was inconsistent among spermatozoa (Fig. 2 a). However, the precise nature and role of this structure remain unclear. We also acknowledge the limitations of the current imaging approach and the morphological variability observed in the micrographs. Further investigation using PNA- or actin-specific cellular stains or anti-tubulin antibodies is warranted to determine whether the projection has an acrosomal, cytoskeletal, or microtubular origin. This will also help rule out the possibility that this structure might be a visual artifact caused by debris or contamination from the spermatophore.

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 to 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 sperm 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 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 fertilize 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 Jr. et al., 2019; Marc et al., 2024). 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 Jr. 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. 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 a commercial redclaw hatchery. Validation of this concept, however, requires breeding studies to investigate the effects of these variables on fertility, as our lab has recently conducted in barramundi (Marc et al., 2024) and has been demonstrated in other vertebrate species (Paul et al., 2008; Didion et al., 2009).

The negative association observed between sperm DNA fragmentation and spermatophore weight in this study may be attributed to the

protective role that heavier spermatophores provide, effectively shielding spermatozoa from environmental stressors such as microbial invasion, reactive oxygen species (ROS) or water quality fluctuations during external fertilization. In crustaceans like lobsters and crayfish, where spermatozoa are aflagellate and non-motile, spermatophores serve as the primary vehicle for sperm transfer (Subramoniam, 2017b). Histochemical analyses have identified high concentrations of acidic mucopolysaccharides, particularly chondroitin sulphate and hyaluronic acid, within these spermatophores (Radha and Subramoniam, 1985; Subramoniam, 2017b). These compounds are known to maintain sperm viability during epizoic storage on the female's sternum by preventing desiccation and reducing microbial contamination. Chondroitin sulphate contributes to the elasticity and mechanical resilience of the spermatophore, while hyaluronic acid, concentrated in the outer layer, supports spermatophore hardening and enhances resistance to external stressors. Beyond structural functions, both compounds may also contribute to the preservation of sperm DNA integrity upon exposure to the external environment. Supporting this, Campo et al. (2004) demonstrated that chondroitin sulphate and hyaluronic acid possess antioxidant properties capable of mitigating oxidative stress, a known driver of DNA fragmentation through the activation of endonucleases and caspases (Wagner et al., 2018). Consequently, heavier spermatophores in this study may contain higher concentrations of these protective compounds, resulting in reduced DNA damage and improved sperm quality. Although the TUNEL assay used here offers robust detection of DNA fragmentation, future studies incorporating oxidative stress markers, including lipid peroxidation and protein carbonyl content, would enhance the interpretation of antioxidant-mediated protection. Furthermore, expanding this research to include larger samples of both wild and farmed males will help clarify the relationship between spermatophore composition, sperm quality, and male reproductive performance in redclaw crayfish aquaculture systems.

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). This result 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 broader range of body weights (8.6–270.6 g), with spermatophore removed from the 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 captive 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, suggesting that further research in this field is warranted.

Ejaculate volume is often linked to sperm concentration, count, and motility in some species, as larger ejaculates typically contain more sperm and accessory secretions (Gilroy and Litvak, 2019; Górski et al., 2021). In this study, spermatophore weights obtained via electro-ejaculation (6–36 mg) were within the range reported for wild marine shrimp (10–93 mg; Rendón Rodríguez et al., 2007; Vuthiphandchai et al., 2007), and positively correlated with sperm concentration, TPFSC, and negatively with DNA fragmentation (Table 3). Similar associations have been documented in other decapods, where spermatophore size/weight influences sperm quality, melanization, and fertilization outcomes (Leung-Trujillo and Lawrence, 1987; Sato et al., 2008; Braga et al., 2010, 2018; Pérez-Rodríguez et al., 2019; Jiang et al., 2020). For instance, Sato et al. (2008) reported that larger spermatophore volumes in coconut crab (*Birgus latro*) contained higher numbers of spermatozoa. Similarly, spermatophore size has been positively linked to sperm counts in river prawn (*Macrobrachium americanum*; Pérez-Rodríguez et al., 2019) and greater egg fertilization success in spiny lobsters (*Jasus* and *Panulirus* spp.; MacDiarmid and Butler, 1999). In redclaw, heavier spermatophores were associated with higher sperm concentration and TPFSC, and slightly reduced DNA damage (Fig. 4 b – d). Correlation analysis revealed a moderate negative association between sperm concentration and DNA fragmentation (Table 3; Fig. 4 e), aligning with patterns reported in non-decapod species by Yang et al. (2019) and Liu et al. (2023). This relationship may be influenced by spermatophore weight, as heavier spermatophores tend to be associated with higher sperm concentrations and lower levels of DNA fragmentation (Fig. 4 b & d). It is therefore plausible that spermatophore quantity plays a role in determining overall sperm quality. Moreover, the observation that higher sperm concentrations are associated with reduced DNA fragmentation may indicate a biological advantage, potentially leading to improved fertilization success and offspring quality. While these findings are encouraging, we acknowledge that the interpretation remains preliminary. The moderate correlation observed suggests that spermatophore weight alone may be insufficient as a standalone proxy for detailed sperm quality analysis in this dataset. Further research with a larger number of biological replicates is recommended to validate and strengthen these associations.

Melanised spermatophores yield low sperm counts and an increase in abnormal spermatozoa in marine shrimps (Braga et al., 2018). Deterioration of spermatophore quality, such as melanisation and a decrease in weight over time, causes a decline in sperm quality and indicates reproductive exhaustion (Leung-Trujillo and Lawrence, 1987; Braga et al., 2010; Jiang et al., 2020). While we did not directly observe evidence of melanisation in wild-caught males used in our study, evaluating the degree of melanisation in redclaw spermatophores may offer an initial screening tool to predict poor sperm quality for inclusion/exclusion of males in more comprehensive downstream diagnostic tests.

The TPFSC calculation provides an integrated assessment of the sperm population capable of promoting successful embryo development, combining both the proportion of viable sperm and the extent of DNA fragmentation. The term "potential fertile" is used in this study to reflect that while TPFSC captures key biomarkers, additional functional traits, including mitochondrial activity and acrosome integrity, may also influence fertilizing capacity but remain to be optimized in redclaw crayfish. This represents the first initiative by our research team to propose and apply this metric in crustacean aquaculture, and, to our knowledge, it constitutes a novel contribution to the assessment of sperm quality in redclaw crayfish. Although references explicitly using the term TPFSC are limited, the conceptual foundation aligns with established principles in animal andrology. Graham et al. (1980, 1990) emphasized that no single laboratory sperm assay reliably predicts fertility, but that combining multiple measures improves predictive accuracy. Similarly, Chenoweth (2022) introduced the concept of Effective Sperm Dose, underscoring the need to evaluate spermatozoa through a composite of functional attributes rather than isolated parameters. In this context, TPFSC serves as a robust integrative indicator, offering a more accurate evaluation of male reproductive potential than sperm concentration alone. We recommend further validation of TPFSC in future studies with larger sample sizes to refine its utility in broodstock selection and improve breeding strategies in redclaw aquaculture.

This study demonstrates that the applied methodologies, including fluorescent staining, flow cytometry, and the novel TPFSC metric, can reliably assess sperm quality and male potential fertility in redclaw crayfish. These tools offer practical value for broodstock evaluation and hatchery management. Future validation in cultured populations may further support their application in selective breeding, cryopreservation, and artificial fertilization to enhance hatchery outcomes and biosecurity in redclaw aquaculture.

5. Conclusions

This study established baseline data on sperm quality in wild-caught *Cherax quadricarinatus* and validated new methodologies for assessing viability and DNA fragmentation via dual fluorescent staining and flow cytometry. Alongside traditional metrics of concentration and morphology, advanced biomarkers provided a refined assessment of potential fertility, including the novel metric of total potential fertile sperm concentration (TPFSC). These tools show promise for predicting male fertility and supporting broodstock selection in redclaw aquaculture.

Future research should enhance these diagnostics by incorporating additional functional indicators, such as acrosomal integrity and mitochondrial function, and validate them through controlled fertility trials that link sperm profiles to reproductive outcomes. Finally, the observation of a putative fertilization-spike structure in some spermatozoa highlights a potentially morphological feature that warrants further study.

CRediT authorship contribution statement

Jon Irish Legaspi Aquino: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lisa Elliott:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Jodie Morris:** Writing – review & editing, Visualization, Validation, Resources, Methodology. **Rhondda Jones:** Writing – review & editing, Visualization, Validation, Software, Formal analysis, Data curation. **Chaoshu Zeng:** Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **John Cavalieri:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Damien B. B.P. Paris:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Jon Irish Legaspi Aquino reports financial support, administrative support, and equipment, drugs, or supplies were provided by Cooperative Research Centre for Developing Northern Australia. Jon Irish Legaspi Aquino reports financial support, administrative support, article publishing charges, equipment, drugs, or supplies, statistical analysis, travel, and writing assistance were provided by James Cook University. Jon Irish Legaspi Aquino reports financial support, administrative support, and equipment, drugs, or supplies were provided by Australian Crayfish Hatchery. Jon Irish Legaspi Aquino reports financial support, administrative support, equipment, drugs, or supplies, statistical analysis, travel, and writing assistance were provided by James Cook University College of Public Health Medical and Veterinary Sciences. Jon Irish Legaspi Aquino reports administrative support and equipment, drugs, or supplies were provided by James Cook University Australian Institute of Tropical Health and Medicine. Jon Irish Legaspi Aquino reports a relationship with Cooperative Research Centre for Developing Northern Australia that includes: funding grants, non-financial support, and travel reimbursement. Jon Irish Legaspi Aquino reports a relationship with James Cook University College of Public Health Medical and Veterinary Sciences that includes: funding grants, non-financial support, and travel reimbursement. Jon Irish Legaspi Aquino reports a relationship with James Cook University that includes: funding grants and non-financial support. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.743494.

Data availability

Data will be made available on request.

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