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Characterization of sperm quality in captive-bred barramundi (*Lates calcarifer*): Effect on spawning performance and paternal contribution in mass-spawning events

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ABSTRACT

Barramundi (Asian sea bass, *Lates calcarifer*) is a species with a mass-spawning reproductive strategy whereby adults synchronously release their gametes into the water column. In captivity, this reproductive strategy usually results in uneven paternal contributions to each cohort of progeny, creating skews in family size that hinder the effectiveness of selective breeding programs. As sperm quality influences fertilization success and early larval development, we investigated the relationship between sperm quality and spawning performance. Accordingly, we assessed three established breeding cohorts. Sperm samples ($n = 22$) were collected by cannulation. Sperm motility was assessed using CASA, and sperm viability and DNA integrity were evaluated using dual-fluorescence stains by flow cytometry. Broodstock were induced to spawn across two consecutive nights, and offspring were collected at 2.5 h and 12 h post-fertilization (hpf) and 24 h and 48 h post-hatching (hph). Offspring were assessed for morphological abnormalities, and key morphological parameters were recorded. Offspring collected at 2.5 hpf and 24 hph were genotyped to determine their parentage and examine the relationship between sperm quality of individual males and offspring survival. Results highlighted that male condition factor (K) and sperm quality were highly variable within each breeding cohort. Males with a lower condition factor showed lower sperm motility and velocity. In contrast, males with a higher condition factor showed higher sperm motility and velocity but also higher levels of sperm DNA damage. While all males were capable of fertilization, males with a lower condition factor had greater paternal contribution. In this study, a reduction in skewed paternity was also found on Night 2 of spawning compared to Night 1. Moreover, males from Tank B, which had the lowest level of sperm DNA damage, had the most even paternal contribution. Conversely, highly skewed maternal contributions were reported across all spawning events. The results of this study suggest that additional complex dynamics and further unmeasured variables, such as spawning behavior, social hierarchy, and the spawning induction procedure, may also skew family sizes. Therefore, further development of artificial reproductive technology is recommended to gain greater control over individual contribution and overcome current breeding bottlenecks.

1. Introduction

Barramundi (Asian sea bass; *Lates calcarifer*) is a species of commercial importance in Australia, with an annual production of 9000 t in 2018–2019 (FRDC, 2020). Barramundi is also recognized as an emerging species on the global stage, with production exceeding 105,800 t in 2020 (FAO, 2022). Despite well-established aquaculture practices in Australia, many producers face breeding and seed stock

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supply challenges. Barramundi has a reproductive strategy whereby males and females synchronously release their gametes in a coordinated mass-spawning event. In aquaculture, spawning events are conducted to accommodate this mass-spawning behavior through hatcheries, placing broodstock together in a large tank with a sex ratio of one female to several males. One of the main challenges of using the mass-spawning breeding strategy is that hatcheries have limited control over the outcome, including individual broodstock contributions. As reported in many mass-spawning species of commercial importance (Borrell et al., 2011; Herlin et al., 2008; Hoskin et al., 2015; Rhody et al., 2014; Sekino et al., 2003), captive-bred barramundi show a highly skewed paternal contribution to progeny cohorts (Domingos et al., 2014; Frost et al., 2006; Liu et al., 2012; Loughnan et al., 2013). Despite having several males in the spawning group, some studies have reported that one or two males often dominated paternity (Frost et al., 2006; Loughnan et al., 2013; Wang et al., 2008). For instance, Frost et al. (2006) found a single male from a cohort of six sired up to 55% of progeny in the first mass spawning. Likewise, in the second mass spawning, one male and one female contributed to 77% and 80% of the total number of progeny, respectively. Furthermore, skewed paternal contribution may be accentuated in captive-bred individuals, as in comparison, mass-spawning events involving wild-caught barramundi broodstock showed a more even paternal contribution (Wang et al., 2008). Such skewing also reduces the amount of genetic diversity captured and passed on to the next generation, threatening the long-term viability of domesticated lines and hindering the effectiveness of selective breeding programs (Brown et al., 2005; Domingos et al., 2014; Loncar et al., 2014; Loughnan et al., 2013). Therefore, it is necessary to investigate the origin of skewed paternity among captive-bred individuals to overcome these bottlenecks.

To date, the causes of skewed paternity have not been assessed in barramundi, and the underlying drivers of spawning outcomes are poorly understood (Domingos et al., 2014; Frost et al., 2006; Liu et al., 2012; Loughnan et al., 2013; Wang et al., 2008). However, one potential cause of the differential paternal contribution observed in captive-bred barramundi could be linked to variation in the sperm quality of males within the breeding cohort. Specifically, the evaluation of fertility in several fish species of commercial importance has highlighted natural inter-male variability in sperm quality, including sperm concentration, motility, and DNA integrity (Beirão et al., 2009; Billard et al., 2004; Cabrita et al., 2006; Gage et al., 2004; Tuset et al., 2008; Vladić et al., 2002). Furthermore, the sperm genome and epigenome have been shown to play an essential role in embryonic and larval development (see review by Herráez et al., 2017). In particular, it has been demonstrated that sperm DNA damage decreases overall reproductive success and compromises early larval development in many fish species (Cabrita et al., 2005a, 2005b; Dziewulska et al., 2011; Gosálvez et al., 2014; Pérez-Cerezales et al., 2010; Zilli et al., 2003).

In commercial barramundi hatcheries, the current measures of sperm quality include a simple visual assessment of sperm motility and milt color. It is mainly performed to ensure males have not changed sex to females between spawning events, as barramundi is a protandrous hermaphroditic species (Budd et al., 2022; Moore, 1979). The absence or limited evaluation of the male reproductive condition is also applied when selecting new males to replace those who have changed sex, potentially allowing naturally sub-fertile individuals to be introduced into the breeding cohort (Frost et al., 2006; Loughnan et al., 2013). With the recent optimization of advanced sperm function assessments in barramundi (Marc et al., 2021), it is now possible to reliably quantify sperm quality and investigate the association between sperm quality and the paternity success of progeny.

Therefore, to inform ongoing breeding practices and aid the selection of male broodstock, the aims of this study were (i) to characterize the variation in sperm quality between male broodstock, (ii) to assess the relationship between sperm quality and paternity success of progeny resulting from mass-spawns, and (iii) to determine the relationship between sperm quality and early larval development in barramundi.

2. Methods

2.1. Animals

Captive-bred barramundi were maintained at the Marine and Aquaculture Research Facility (MARF), James Cook University, Townsville, Queensland, Australia. Males were two to four years old, and females were five to eight years old. Fish were held in breeding cohorts at a sex ratio of 1:2 (female:male) in three separate recirculated systems (A, B, and C) maintained at 30 °C, salinity 30 ppt, and 16 h light:8 h dark cycle. Tank A and B volumes were 28,000 L and contained eight males and four females each, whereas Tank C volume was 18,000 L and contained six males and three females (Table 1). Fish were fed a formulated diet four days a week at 1% body weight (LANSY-Breed Essential, INVE Technologie, Belgium). Broodstock were identified by their unique Passive Integrated Transponder (PIT; Hallprint Pty. Ltd., Hindmarsh Valley, Australia) tags. Experiments were approved by the James Cook University Animal Ethics Committee (A2406).

2.2. Sperm collection and assessment

Male broodstock were sedated in a saltwater bath containing 40 ppt AQUI-S®(AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) to facilitate handling and sperm collection. Total length (TL) and body weight (BW) were recorded. Fulton's body condition factor $K = \frac{BW}{TL^3}$ was calculated (Froese, 2006). Sperm samples were obtained by genital cannulation using a 1.72 mm diameter cannula (PE-90 polyethylene tubing, Becton Dickinson, Sparks, MD, USA; Schipp et al., 2007). The milt volume was determined by measuring the length of raw sperm in the cannula, as described by Marc et al. (2021). Sperm samples were assessed for motility directly after collection at the broodstock facility. The remaining sperm samples were held on ice for ~3 h before being analyzed for viability and DNA damage at the laboratory using flow cytometry.

2.3. Sperm quality

2.3.1. Sperm concentration and motility

Sperm concentration and sperm motility assessments were conducted using the computer-assisted sperm analyzer (CASA) system, according to Marc et al. (2021). In short, the CASA system consisted of a light microscope (Olympus CX41; Olympus, Tokyo, Japan) equipped with a 20 X negative phase-contrast objective, 0.5 X C-Mount adaptor, and a Basler avA1000-100gc high frame-rate area scan camera (Basler AG, Ahrensburg, Germany), coupled with the CASA software (AndroVision, Minitüb GmbH, Tiefenbach, Germany). The AndroVision® software was calibrated to detect barramundi spermatozoa using the following settings: *color thresholds*, 118 to 255 (red and green) and 0 to 255 (blue); *object appearance*, middle; *object features*, 3.5 to 35 area, and 0.5 to 3.5 form; *halo filter*, on; *camera calibration*, 1 pixel = 0.54 μm; *field of view*, 555.12 μm × 555.12 μm. The sperm assessment was conducted in triplicate for each fish and assessed upon activation in natural saltwater (i.e., 30 ppt) using a 1:1000 dilution ratio. Leja 4-chamber linear-flow slides (Leja Products B. V., Nieuw Vennep, Amsterdam, Netherlands) pre-warmed at 30 °C were used to contain sperm samples during analysis. Spermatozoa were evaluated 15 s after activation by videos recorded for 1 s at a frame rate of 60 fps.

The AndroVision® *Sperm Motility* module enabled the simultaneous determination of sperm concentration and motility. Sperm concentration was determined using the following equation: $[c] = \frac{N}{V} \times$ *dilution factor* , where the number of spermatozoa per field of view (N) was divided by the fluid volume of Leja chamber contained in each field of view (V) that is 6.16×10^{-6} *ml*, and multiplied by the dilution factor.

Table 1
Description of barramundi (*Lates calcarifer*) broodstock cohorts per tank.

Tank	Total number of fish/tank	Total number of females/ males	Female: male sex ratio	Male mean weight (kg)	Male mean length (cm)	Male mean condition K	Female mean weight (kg)	Female mean length (cm)	Female mean condition K	Stocking density (kg/ m^3)
A	12	4/8	1:2	4.0 ± 0.3	70.7 ± 0.6	1.14 ± 0.07	9.0 ± 0.4	86.1 ± 1.0	1.43 ± 0.14	2.4
B	12	4/8	1:2	2.9 ± 0.3	67.2 ± 0.4	0.94 ± 0.04	7.3 ± 0.3	86.0 ± 0.4	1.15 ± 0.04	1.9
C	9	3/6	1:2	3.5 ± 0.2	73.1 ± 0.4	0.90 ± 0.04	8.3 ± 0.2	88.5 ± 0.5	1.21 ± 0.07	2.6

Data are displayed as mean ± standard error.

Sperm motility parameters, including total motility (TM; %), progressive motility (PM; %), slow, medium, and fast motility (%), curvilinear velocity (VCL; μm/s), average path velocity (VAP; μm/s), straight-line velocity (VSL; μm/s), the amplitude of lateral head displacement (ALH; μm), straightness of the average path (STR; % of VSL/VAP), the linearity of the curvilinear path (LIN; % of VSL/VCL), wobble (WOB; % of VAP/VCL), and beat-cross frequency (BCF; Hz) were evaluated. Sperm motility categories were classified based on VCL thresholds, including total motility (VCL ≥ 15 μm/s), progressive motility (VCL ≥ 35 μm/s), slow motility (VCL ≥ 15 and < 35 μm/s), medium motility (VCL ≥ 35 and < 100 μm/s), and fast motility (VCL ≥ 100 μm/s).

2.3.2. Sperm viability

Sperm viability was performed following the method described by Marc et al. (2021) and assessed using a dual-staining Hoechst 33342/ Propidium Iodine (PI) method. In brief, sperm concentration was standardized to 100 × 10^6 sperm/mL. Each sperm sample was aliquoted to generate an unstained control (U1), single- (P1) and double-stained (P2) positive controls, and a test sample. Positive controls were incubated at 70 °C for 5 min to rupture the sperm plasma membrane. Samples were each incubated at room temperature in the dark for 30 min in 10 μg/mL Hoechst 33342 (P2 and test samples), then incubated for a further 7 min in the dark in 10 μg/mL PI (P1, P2, and test samples). All samples were then resuspended in 400 μL in PBS and directly analyzed by flow cytometry. The analysis of each sample was performed in triplicate.

2.3.3. Sperm DNA integrity

DNA integrity using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL - FITC/PI) assay was performed following the method described by Marc et al. (2021). Sperm concentration was standardized to 100 × 10^6 sperm/mL. Unlabeled control (U1), unlabeled control with PI stain (U2), negative control in label solution (N1), negative control in label solution with PI (N2), DNase-treated FITC-positive control (P1), and DNase-treated FITC-positive control with PI (P2) were generated for each sperm sample. Controls and test samples were centrifuged at 320*g* for 5 min, and each pellet was fixed in fresh 2% paraformaldehyde in PBS for 1 h at room temperature. Next, the sample was washed in PBS at 320 g for 5 min, and the pellet was resuspended in 100 μL fresh 0.1% Triton X-100 in 0.1% sodium citrate solution for 2 min at 4 °C. Control and test samples were washed in PBS at 320 g for 5 min. Positive controls were resuspended in 100 μL of 1000 U/mL DNase 1 in Roche Buffer 2 and incubated for 10 min at room temperature. Control and test samples were resuspended in the appropriate solution: unlabeled controls (U1 and U2) in 50 μL PBS, negative controls (N1 and N2) in 50 μL TUNEL labeling solution without enzyme, and positive controls (P1 and P2) and each test sample in 50 μL of TUNEL reaction mixture containing enzyme. All samples were incubated in the dark for 60 min at 37 °C in the dark. Next, U2, N2, P2, and all test samples were counterstained in 10 μg/mL PI and incubated in the dark for 7 min at room temperature. Control and test samples were then resuspended in 400 μL PBS and directly analyzed by flow cytometry. The analysis of each sample was performed in triplicate.

2.3.4. Flow cytometry

Dual fluorescence-stained spermatozoa were measured by FACS Canto™ II flow cytometry (8-color; blue/red/violet lasers; serial number: V33896202614; manufactured May 2016; BD Biosciences, San Jose, CA, USA). Control quality beads (Lot: 7130918, BD FACSDiva™ CS&T Research Beads, San Jose, CA, USA) were used to calibrate the three lasers before each analysis. Blue Hoechst fluorescence was measured using the 405 nm laser and 450/50 nm filter; green FITC fluorescence was measured using the 488 nm laser and 530/30 nm filter; red PI fluorescence was measured using the 488 nm laser and 610 nm LP mirror. Detector voltages were set to FSC = 549 V, SSC = 401 V, Hoechst filter = 412 V, FITC filter = 469 V, and PI filter = 543 V. Data were analyzed using FlowJo software (Treestar, Inc., San Carlos, CA, USA).

2.4. Spawning induction and egg collection

Following gamete collections, sedated broodstock were subject to a single intramuscular injection of luteinizing hormone-releasing analog (LHRHa; Syndel International, Vancouver, British Columbia, Canada) to trigger spawning events (Thépot and Jerry, 2015). Males received an LHRHa injection of 25 μg/kg, and females with mature oocytes (>350 μm visually estimated under a microscope) were selected and received an injection of 50 μg/kg (Guppy et al., 2022; Thépot and Jerry, 2015). The administration of LHRHa triggered two consecutive spawning events on the second and third nights post-injection. Buoyant fertilized eggs were collected from a 500 L egg collector tank containing a 250 μm mesh by a surface skimmer outlet connected to the main tank.

Offspring were sampled at four critical developmental phases: (i) 2.5 h post-fertilization (hpf; 1 k cell stage – the start of zygotic transcription/ organ development) in the egg collector tank, and (ii) 12 hpf (hatching, free-swimming stage), (iii) 24 h post-hatch (hph; eyes developed, using yolk sac reserve), and (iv) 48 hph (yolk sac depleted, mouth-formed, first feeding) in the hatch tanks (Thépot and Jerry, 2015; Walford and Lam, 1993).

Once the first sampling point at 2.5 hpf was completed, spawning success was assessed as per current commercial practices, including total egg count, fertilization rate, hatching rate, and survival rates at 24 and 48 hph. Specifically, once offspring reached the germ-ring stage (i.e., 3–5 hpf), eggs were collected with a 250 μm net and transferred into a 10 L bucket. Eggs in the bucket were gently homogenized while five aliquots of 1 mL were taken. The total egg count and fertilization rate were determined based on the average of embryos counted from the five aliquots using a Sedgewick rafter counting cell. Eggs were transferred to 500 L hatch tanks and stocked as per commercial practices (Schipp et al., 2007). Larvae were collected for subsequent samplings at 12 hpf, 24 hph, and 48 hph. Specifically, larvae in the hatch tanks were gently homogenized while five aliquots of 40 mL were taken. The hatching rate and survival rates at 24 and 48 hph were determined based on the total number of live and dead larvae in each hatch tank. The larval survival rates at 24 and 48 hph are expressed sequentially to indicate the proportion of larvae that survived from the previous stage and cumulatively to show the proportion of larvae that survived since fertilization.

For parentage analysis, the developmental stage of the eggs (1 k cell stage) was verified before being preserved in 70% ethanol. Three

distinct samples (i.e., ~300 eggs each) were taken as biological replicates for each time point. Based on the spawning success data, parentage analysis was performed on offspring collected at 2.5 hpf and 24 hph to investigate potential shifts in paternal contribution before and after the drop in larval survival occurring during this timeframe.

2.5. Assessment of offspring phenotype

Eggs and larvae collected at the four critical developmental phases were viewed under a stereomicroscope (Olympus SZX10; Olympus, Tokyo, Japan) coupled with a digital camera (Basler acA2040-120uc, Basler AG, Ahrensburg, Germany). Micrographs were captured, and egg and larval morphological features were measured following the method described by Guppy et al. (2022). The morphological features measured for eggs were egg size, egg roundness, oil droplet size, and oil droplet roundness. Egg and oil droplet sizes were defined at the Feret's diameter or maximum diameter (μm, ± 0.1 μm) between any two points on the feature boundary. Egg roundness was calculated as $Roundness = (4 \times area)\left(\pi \times Egg\ diameter^2\right)$, with values approaching 1.00, indicating a perfectly round egg. For larvae, morphological features were measured, including total length (TL, ± 1 μm), yolk sac Feret's diameter (YS, ± 1 μm), and oil globule Feret's diameter (OG ± 1 μm) at 12 hpf, and TL, OG, and eye diameter (ED, ± 1 μm) at 24 hph and 48 hph. The morphological features were measured using ImageJ analysis software (Version 1.41; http://rsbweb.nih.gov/ij/).

2.6. DNA extraction and genotyping

Each embryo/larva was allocated to a single well in a 94-well plate for DNA extraction to determine individual parent–progeny relationships. DNA extraction and PCR amplification of microsatellites were performed according to Domingos et al. (2013). In brief, the DNA of individual embryo/larva was extracted using Tween®-20 lysate buffer and proteinase K. Once digested, plates were centrifuged, and the supernatant of the crude tissue lysate was used as a genomic DNA (gDNA) template for subsequent PCR. Broodstock genotype data were provided by Mainstream Aquaculture Group Pty Ltd. Offspring were genotyped using two multiplex suites totaling 16 loci, one containing nine microsatellite loci (Lca008, Lca020, Lca021, Lca058, Lca064, Lca069, Lca70, Lca074, and Lca098; Zhu et al., 2006) and the other containing seven microsatellite loci (Lca003, Lca016, Lca040, Lca057, Lca154, Lca178, and Lca371; Domingos et al., 2014). PCR reaction and cycling conditions, allele scoring, and fragment analysis were performed according to established protocols (Domingos et al., 2014, 2013; Loughnan et al., 2013). The PCR mixture was composed of 1 μl of 10 x primer mix, 5 μl of MyTaq™HS Mix (Bioline, Eveleigh, NSW, Australia), 3.5 μl of water, and 0.5 μl of the lysate DNA. Microsatellite amplification was performed on a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the following cycling conditions: 95 °C for 5 min (initial denaturation) followed by ten cycles of 95 °C for 30 s (denaturation), 57 °C for 90 s (annealing) and 72 °C for 30 s (extension), then 20 cycles of 95 °C for 30 s (denaturation), 55 °C for 90 s (annealing) and 72 °C for 30 s (extension), then a final step of 60 °C for 30 min. The success of PCR amplification was checked by visualization on a 1.5% TBE agarose gel containing GelGreen® (Biotium Inc., Hayward, CA, USA), where 2 μL of PCR product was loaded and electrophoresed for 25 min at 80 V and 400 mA. The remaining PCR products were diluted with 12 μL of water and desalted through a Sephadex® G-50 Fine (GE Healthcare, Uppsala,

Sweden) filtration spin column for 3 min at 930 g. The Sephadex cleaning process was confirmed by visualization on a 2% TBE agarose gel, where 2 μL of DNA with 2 μL of Orange G dye and 1 μL of Easyladder 1 for reference were loaded and electrophoresed for 30 min at 90 V. A total of 94 larvae were processed for each spawning night and time point and sent to the Australian Genome Research Facility (AGRF), Melbourne, Australia, for genotyping.

Microsatellite genotyping was performed using fragment separation analysis using an Applied Biosystems (ABI) 3730 DNA system. A size standard, GeneScan 500 LIZ Ladder (Applied Biosystems, Foster City, CA, USA), was included with each PCR sample. Positive and negative controls were run simultaneously to ensure allele scoring was consistent and verify that no apparent contamination had occurred during laboratory protocols. Samples with four or more failed alleles were considered unsuccessful and excluded from the sample data set. All allele data files were analyzed using Genemarker 2.4.0 software (Soft Genetics, State College, Pennsylvania, USA).

2.7. Parentage assignment

Genotypic data were assessed for scoring errors introduced by allele stutter and the presence of null alleles using MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004). Genotyping was considered successful when scoring PCR products provided size information for at least seven markers per sample (Domingos et al., 2013). Parentage analysis was performed using CERVUS 3.0.3 (Kalinowski et al., 2007) to determine each male's contribution to a cohort of offspring and the total number of half and full-sibling families within each spawning event. A strict confidence level (CI) of 95% was utilized to determine the most likely parent pair assigned to the offspring. Microsatellite data were formatted to GENPOP and Fstat file structure using GenAlEx 6.51b2 (Smouse et al., 2017). The allelic richness (A_r) was calculated using Fstat Version 2.9.3.2 (Goudet, 2002). The mean number of alleles per locus (k), the observed heterozygosity (H_o), the expected heterozygosity (H_e), the inbreeding coefficient (F_{is}), and the deviation observed from expected proportions under Hardy-Weinberg equilibrium (HWE) were determined using GenoDIVE version 3.0 (Meirmans, 2020). The effective numbers of dams $N_{ed} = \frac{N_d K_d - 1}{\left[K_d - 1 + \left(\frac{V_d}{K_d}\right)\right]}$ and sires $N_{es} = \frac{N_s K_s - 1}{\left[K_s - 1 + \left(\frac{V_s}{K_s}\right)\right]}$, and the effective population size were calculated according to Frankham et al. (2002), where N_d and N_s were the numbers of dams and sires respectively, K_d and K_s were the mean numbers of offspring per dam and sire, and V_d and V_s were the variance in the contribution for dams and sires. The rate of inbreeding $\Delta F = \frac{1}{2N_e}$ was calculated according to Falconer (1989).

2.8. Statistical analysis

Statistical analyses were performed using RStudio version 1.0.153 (RStudio, Inc., Boston, MA, USA). Normal distribution of data and homogeneous variance were assessed using Shapiro-Wilk and Levene tests. Where data were not normally distributed or had heterogeneous variance, data transformations were undertaken to allow the assumptions of subsequent statistical tests to be met. Specifically, variables including condition factor, fast motility, sperm viability, sperm DNA damage, and paternal contribution at 2.5 hpf and 24 hph were transformed with the logit function, and sperm velocities (i.e., VCL, VSL, and VAP) were $\log_{10}$ transformed. Pearson correlations were used to measure the relationship between broodstock physical traits (i.e., BW, TL, and condition factor), milt characteristics (i.e., volume and sperm concentration), sperm parameters (i.e., TM, PM, slow, medium, fast, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF, live, and DNA damage), and paternal contribution at 2.5 hpf and 24 hph. Principal component analysis (PCA) was used to investigate the relationships between the total variation observed and the variables measured, including BW, TL, condition factor, milt volume, sperm concentration, sperm motility (i.e., TM, VCL, VSL, VAP, ALH, and BCF), sperm integrity (i.e., viability and DNA damage), and paternal contribution at 2.5 hpf and 24 hph. Values for each of the variables were mean-centered and scaled. The number of PCs was determined based on the number of PCs that contributed to a preselected total of explained variation of 80%. Embryo and larval morphometries were compared across spawns with one-way ANOVA,

followed by Tukey's multiple comparison test. A Kruskal-Wallis rank-sum test was performed when data were not normally distributed and variances homogeneous, followed by Dunn's test for multiple comparisons to assess the significance between spawning nights. Data are displayed as mean ± standard error (SEM). The level of significance was set at $P \leq 0.05$.

3. Results

3.1. Characterizing baseline variation in broodstock condition and sperm quality

Male barramundi broodstock morphometrics were BW 3.5 ± 0.2 kg, TL 70.1 ± 1.0 cm, and condition factor 1.00 ± 0.04. While variations in physical traits were present between males, the condition factor ranged between 0.71 and 1.19, except for one male M2 with a condition factor of 1.59 (Table 2). Milt characteristics, including milt volume (CV = 62.5%), sperm concentration (CV = 36.9%), and total count (CV = 82.2%), were highly variable across the broodstock (Table 2). Similarly, the proportion of motile spermatozoa (TM: CV = 37.5%, PM: CV = 45.5%, and fast motility: CV = 80.3%) varied considerably between individuals (Table 2). In contrast, parameters for sperm swimming trajectory (LIN, STR, WOB, ALH, and BCF) were relatively consistent between males (CV < 25%; Table 2), while parameters for sperm velocity (VSL: CV = 50.6% and VAP: CV = 31.6%), showed higher variation (Table 2). Of note, M2 with the highest condition factor had low milt volume and sperm concentration but highest proportion of motile sperm (TM: 70.1 ± 2.3%, PM: 60 ± 7.8%, and Fast: 46.1 ± 10.6%), sperm velocities (VCL: 152.9 ± 27.2 μm/s, VSL: 128.3 ± 24.3 μm/s, and VAP: 147.3 ± 29.2 μm/s), and straightest swimming trajectory (LIN: 74.0 ± 6.0%, STR: 82.0 ± 3.0%, WOB: 87.0 ± 6.0%, ALH: 0.48 ± 0.01 μm, and BCF: 13.8 ± 0.71 Hz; Supplementary Table 1). Sperm viability across individuals was high overall, with only 15.6 ± 1.8% of the total spermatozoa population considered dead (Table 2). Sperm DNA damage was >1% for 11 out of 22 males, with three males observed with extremely high levels of DNA damage (M5: 19.3 ± 0.3%, M6: 24.7 ± 0.1%, and M7: 58.9 ± 0.9%), resulting in high variation between males (CV = 207.7%; Table 2; Supplementary Table 1).

Table 2
Physical condition, milt characteristics, and sperm quality of captive-bred male barramundi (*Lates calcarifer; n* = 22) broodstock.

Parameter	Mean ± SEM	Median	Min	Max	CV (%)
Broodstock condition					
Weight (kg)	3.5 ± 0.2	3.35	1.9	4.9	20.9
Length (cm)	70.1 ± 1.0	70	59.0	76.5	6.8
Condition K	1.00 ± 0.04	0.99	0.72	1.59	17.6
Milt characteristics					
Volume (μL)	33.1 ± 4.4	30.6	3.5	69.7	62.5
Concentration (x 10^9 sperm/mL)	19.2 ± 1.5	21.0	5.5	31.7	36.9
Total count (x 10^6 sperm)	731.7 ± 128.1	524.5	35.0	2023.3	82.2
Sperm motility[a]					
Total motility (%)	36.0 ± 2.9	36.2	13.4	70.1	37.5
Progressive motility (%)	26.0 ± 2.5	25.8	9.2	60.0	45.5
Slow motility (%)	10.0 ± 0.7	10	1.1	15.4	31.4
Medium motility (%)	14.1 ± 1.2	13.9	3.3	25.0	38.5
Fast motility (%)	11.7 ± 2.0	9.7	2.4	46.1	80.3
VCL (μm/s)	83.3 ± 4.5	79.3	52.2	152.9	25.5
VSL (μm/s)	47.0 ± 5.1	42.7	19.6	128.4	50.6
VAP (μm/s)	72.4 ± 4.9	68.2	36.2	147.3	31.6
LIN (%)	50.9 ± 2.6	52.0	33.0	74.0	24.3
STR (%)	64.8 ± 2.3	68.0	48.0	82.0	16.8
WOB (%)	75.5 ± 1.5	76.5	60.0	88.0	9.2
ALH (μm)	0.53 ± 0.01	0.51	0.42	0.67	12.5
BCF (Hz)	12.2 ± 0.4	11.9	8.7	16.1	15.7
Sperm integrity					
Live (%)	84.4 ± 1.8	85.9	60.7	93.8	10.2
DNA damage (%)	6.5 ± 2.9	0.7	0.01	58.9	207.7

Data are displayed as mean ± standard error (SEM). *n*, number of fish; Total motility (VCL ≥ 15 μm/s); Progressive motility (VCL ≥ 35 μm/s); Slow motility (VCL ≥ 15 and < 35 μm/s); Medium motility (VCL ≥ 35 and < 100 μm/s); Fast motility (VCL ≥ 100 μm/s); VCL, curvilinear velocity (μm/s); VSL, straight-line velocity (μm/s); VAP, average path velocity (μm/s); LIN, linearity (%); STR, straightness (%); WOB, wobble (%); ALH, the amplitude of lateral head displacement (μm); BCF, beat cross frequency (Hz); Live, the proportion of viable sperm (%); DNA damage, the proportion of DNA damaged sperm (%).
[a] see Supplementary Table 1 for sperm motility data per fish.

3.2. Relationship between physical traits, milt characteristics, and sperm quality parameters

A number of statistically significant relationships were observed between broodstock physical traits, milt characteristics, and sperm quality parameters (Table 3). Specifically, there was a negative correlation between BW and milt volume ($r(22) = -0.46$, $P < 0.05$) and a positive relationship with BW and VSL ($r(22) = 0.42$, $P < 0.05$), LIN ($r(22) = 0.44$, $P < 0.05$), and sperm DNA damage ($r(22) = 0.66$, $P < 0.001$; Table 3). Broodstock length was negatively correlated to sperm motility (TM, PM, and Fast; Table 3), while condition factor showed a negative correlation with milt volume ($r(22) = -0.45$, $P < 0.05$) and sperm concentration ($r(22) = -0.51$, $P < 0.05$) and but a positive correlation with Fast, VCL, VSL, and VAP (Table 3).

Milt volume was strongly correlated with sperm concentration ($r(22) = 0.70$, $P < 0.001$) and moderately correlated to medium motility ($r(22) = 0.47$, $P < 0.05$), while also negatively correlated to sperm velocities (i.e., VCL, VSL, and VAP; Table 3). Sperm concentration was positively correlated to slow motile spermatozoa ($r(22) = 0.58$, $P < 0.01$) and negatively correlated to sperm velocities (i.e., VCL, VSL, and VAP) and swimming trajectory parameters (i.e., LIN, STR, and WOB; Table 3).

Sperm motility parameters (i.e., TM and PM) showed a strong correlation with motile sperm subpopulations (i.e., Slow, Medium, and Fast) but were not significantly associated with any other sperm quality variables, including sperm velocities and sperm integrity (Table 3). However, VSL, LIN, and STR were positively associated with sperm viability and DNA damage, WOB to sperm DNA damage ($r(22) = 0.48$, $P < 0.05$), and ALH negatively associated with sperm DNA damage ($r(22) = -0.52$, $P < 0.05$; Table 3).

3.3. Spawning data

Outcomes of the spawns conducted in Tanks A, B, and C were highly variable in terms of the total number of eggs produced (2.08–8.52 million), fertilization rate (5.2–77.5%), and hatching rate (0.0–92.5%; Table 4). Despite these differences, all spawns produced viable batches of larvae without detectable physical deformities, except for Tank A on Night 1, which, in association with a low fertilization rate (mean: 5.2 ± 1.0%), resulted in too few hatched larvae to determine an accurate hatch rate (< 0.001%; Table 4).

Across all spawning events, there were several general trends observed throughout embryonic and larval development. Mortality of progeny mainly occurred between the 1 k-cell stage and 24 h after hatching. Specifically, an initial decline (~25%) was observed during embryo development between 2.5 hpf and hatching (Table 4), and a second decline (~25%) occurred over the next 24 h of larval development across all spawns, except for Tank B on Night 2 (Table 5). Finally, the rate of larval survival (≥ 90.9%;) stabilized across all spawns between 24 and 48 hph, with very few further mortalities observed (Table 5).

Egg size showed significant variation between spawns, with the largest eggs occurring in Tank A and the smallest in Tank C both on Night 1 (ANOVA, $F(5, 294) = 122.7$, $P < 0.001$; Table 6). Similarly, oil droplet size was the smallest in Tank C on both nights (Kruskal-Wallis, H

Table 3
Relationship between physical traits, milt characteristics, and sperm quality parameters of captive-bred male barramundi (*Lates calcarifer*; $n = 22$) broodstock.

	BW	TL	K	Vol.	Conc.	TM	PM	Slow	Medium	Fast	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	Live
BW																			
TL	**0.70*****																		
K	**0.43***	−0.32																	
Vol.	**−0.46***	−0.12	**−0.45***																
Conc.	−0.35	0.02	**−0.51***	**0.70*****															
TM	−0.20	**−0.43***	0.34	0.17	0.07														
PM	−0.17	**−0.45***	0.41	0.10	−0.07	**0.98******													
Slow	−0.19	−0.13	−0.07	0.37	**0.58****	**0.61****	**0.44***												
Medium	−0.30	−0.15	−0.15	**0.47***	0.29	**0.62****	**0.62****	0.30											
Fast	−0.08	**−0.46***	**0.50***	−0.01	−0.12	**0.92******	**0.92******	**0.47***	0.38										
VCL	0.11	−0.30	**0.52***	**−0.43***	**−0.52***	0.27	0.37	−0.24	−0.28	**0.47***									
VSL	**0.42***	0.09	**0.49***	**−0.43***	**−0.60****	0.21	0.31	−0.28	−0.12	0.30	**0.72*****								
VAP	0.20	−0.20	**0.51***	**−0.44***	**−0.53***	0.20	0.30	−0.28	−0.31	0.41	**0.98******	**0.79******							
LIN	**0.44***	0.22	0.38	−0.32	**−0.52***	0.13	0.23	−0.32	0.06	0.16	0.38	**0.88******	0.49*						
STR	0.42	0.27	0.30	−0.22	**−0.48***	0.16	0.25	−0.27	0.19	0.13	0.21	**0.81******	0.32	**0.96******					
WOB	0.33	0.06	0.40	−0.41	**−0.51***	−0.03	0.09	**−0.45***	−0.21	0.12	**0.67*****	**0.80******	**0.77******	**0.81******	**0.64****				
ALH	−0.42	−0.38	−0.15	0.18	0.33	0.05	0.00	0.22	−0.06	0.09	0.09	**−0.59****	−0.03	**−0.84******	**−0.90******	**−0.45***			
BCF	−0.04	0.05	−0.07	−0.01	−0.41	0.14	0.28	**−0.47***	**0.43***	0.09	0.17	0.38	0.16	**0.44***	**0.53***	0.25	−0.32		
Live	0.09	0.17	−0.03	0.37	0.07	0.34	0.36	0.13	0.36	0.27	0.24	**0.55****	0.32	**0.57****	**0.60****	0.41	−0.39	0.36	
DNA	**0.66*****	0.29	**0.47***	−0.41	**−0.61****	−0.24	−0.14	**−0.49***	−0.35	0.01	0.34	**0.61****	0.41	**0.55****	**0.53***	**0.48***	**−0.52***	0.17	0.15

Matrix of Pearson correlation coefficients between all variables. The data were logit transformed for body condition, fast motility, sperm viability, and sperm DNA damage, or $\log_{10}$ transformed for VCL, VSL, and VAP. Abbreviation: BW, body weight; TL, total length; K, body condition; Vol., milt volume; Conc., sperm concentration; TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, the amplitude of lateral head displacement; BCF, beat cross frequency; Live, the proportion of viable sperm; DNA, the proportion of sperm DNA damage. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ denote level of statistical significance.

Table 4
Number (n) of females, total number of eggs produced, egg production per kilogram, and fertilization and hatching rates of barramundi (*Lates calcarifer*) for each breeding cohort and mass-spawning night.

	Tank A		Tank B		Tank C	
	Night 1	Night 2	Night 1	Night 2	Night 1	Night 2
Female n	4	4	4	4	3	3
Eggs produced (x 10^6)	2.08 ± 0.16	4.66 ± 0.2	8.52 ± 0.19	5.90 ± 0.04	5.46 ± 0.39	2.66 ± 0.75
Egg production (x 10^6 kg^{-1})	0.06	0.13	0.29	0.20	0.22	0.10
Fertilization rate (%)	5.2 ± 1.0	77.5 ± 0.7	71.9 ± 4.9	75.1 ± 4.0	52.9 ± 3.7	59.3 ± 1.8
Hatching rate (%)	0.0 ± 0.0	80.4 ± 2.0	92.5 ± 2.6	77.7 ± 2.1	77.7 ± 2.3	46.1 ± 0.5

Data are displayed as mean ± standard error.

(5) = 202.44, $P < 0.001$). However, the roundness of the egg ($\overline{X}$= 0.98 ± 0.001; Kruskal-Wallis, H(5) = 5.31, $P = 0.38$) and oil droplet ($\overline{X}$ = 0.97 ± 0.002; Kruskal-Wallis, H(5) = 9.58, $P = 0.088$) were both consistent across breeding cohorts and nights (Table 6).

The total length of hatched larvae varied significantly between spawns, with the longest larvae occurring in Tank C on Night 2 (ANOVA, F(4, 95) = 35.62, $P < 0.001$; Table 7). The total length of larvae increased by an average of 590 μm 24 h after hatching; however, the differences between spawns remained significant (Kruskal-Wallis, H(4) = 13.37, $P = 0.0096$). Total length plateaued between 24 and 48 hph and still differed significantly between spawns, with the shortest larvae occurring in Tank C on Night 2 (Kruskal-Wallis, H(4) = 48.26, $P <$ 0.001). The yolk sac was largest in larvae from Tank B and smallest from Tank A (Kruskal-Wallis, H(4) = 78.65, $P < 0.001$) and was completely absorbed by 24 hph (Table 7). Once the eyes of larvae were fully formed at 24 hph, average eye size remained similar (~233 μm) at both 24 and 48 hph. However, eye size was significantly larger in larvae from Tank C on Night 2 (24 hph: ANOVA, F(4, 36.99) = 6.98, $P = 0.0003$) and Night 1 (48 hph: ANOVA, F(4, 77) = 4.85, $P = 0.002$; Table 7).

3.4. Parental contribution

The number of offspring successfully genotyped and assigned to a parental pair at 2.5 hpf and 24 hph for each mass-spawning night was high on average (mean: 97.2 ± 0.7%; Supplementary Table 2). All males contributed at least once across the two consecutive spawning nights. In Tank A, seven out of eight males on Night 1 and all males on Night 2 were detected to have contributed to the paternity of progeny at 2.5 hpf. Specifically, M1 and M8 appeared to dominate paternity on Night 1 and M3, M4, and M8 on Night 2 (Fig. 1a; Supplementary Table 3). All males contributed to the paternity of progeny in Tanks B and C across Nights 1 and 2 (Fig. 1b and c; Supplementary Table 3). In Tank B, M11 and M14 appeared to dominate paternity on Night 1, and M9, M12, M13, M14, and M16 seemed to dominate on Night 2. In Tank C, the proportion of male paternity was highly skewed, with up to 54.3% paternity being dominated by M20 at 24 hph on Night 1 and M19 dominating paternity on Night 2 (Fig. 1c; Supplementary Table 3).

Female contribution to the maternity of different cohorts of progeny was inconsistent. F1 from Tank A did not contribute fertilized eggs on either night, while F2 dominated maternity, contributing as much as 95.6% to the progeny (Fig. 1a; Supplementary Table 3). In Tank B, F5 and F6 had limited maternal contributions, while F8 appeared to dominate maternity on both nights (Fig. 1b; Supplementary Table 3). In Tank C, F9 dominated the maternity of progeny on both nights (Fig. 1c; Supplementary Table 3). The number of families detected at 2.5 hpf and 24 hph on Nights 1 and 2 was about half the number of potential families that could have been generated if the parental contribution was evenly

Table 5
Sequential and cumulative larval survival rates at 24 and 48 h post-hatch (hph) of barramundi (*Lates calcarifer*) for each breeding cohort and mass-spawning night.

	Tank A				Tank B				Tank C			
	Night 1		Night 2		Night 1		Night 2		Night 1		Night 2	
Survival (%)	Seq.	Cum.	Seq.	Cum.	Seq.	Cum.	Seq.	Cum.	Seq.	Cum.	Seq.	Cum.
24 hph	NA	NA	61.5 ± 2.1	49.4 ± 1.7	79.4 ± 6.9	73.2 ± 4.3	103.6 ± 0.9	80.5 ± 2.9	73.1 ± 0.1	56.8 ± 1.6	82.3 ± 3.0	37.8 ± 0.8
48 hph	NA	NA	98.8 ± 2.5	48.8 ± 1.2	96.2 ± 2.1	70.6 ± 5.7	97.2 ± 2.6	78.4 ± 4.9	98.8 ± 0.8	56.1 ± 1.1	90.9 ± 3.5	34.4 ± 1.9

Data are displayed as mean ± standard error.

Table 6
Morphometry of barramundi (*Lates calcarifer*) eggs at 2.5 h post-fertilization for each breeding cohort and mass-spawning night.

	Tank A		Tank B		Tank C	
	Night 1	Night 2	Night 1	Night 2	Night 1	Night 2
Egg (*n*)	50	50	50	50	50	50
Egg size (μm)	781.6 ± 2.1^{a}	771.2 ± 2.1^{b}	761.6 ± 2.5^{b}	767.3 ± 2.4^{b}	705.7 ± 2.8^{c}	762 ± 2.5^{b}
Egg roundness	0.98 ± 0.002	0.98 ± 0.002	0.98 ± 0.002	0.98 ± 0.002	0.98 ± 0.002	0.99 ± 0.001
Oil droplet size (μm)	255.1 ± 1.1^{a}	253.5 ± 1.2^{a}	256.5 ± 1.3^{a}	238.8 ± 2.2^{b}	221.3 ± 1.6^{c}	208.5 ± 2.4^{c}
Oil droplet roundness	0.97 ± 0.004	0.96 ± 0.004	0.97 ± 0.003	0.97 ± 0.003	0.97 ± 0.003	0.97 ± 0.004

Data are displayed as mean ± standard error. *n,* the number of eggs measured. Different letters indicate significant differences ($P < 0.05$).

Table 7
Morphometry of barramundi (*Lates calcarifer*) larvae at hatching and 24 and 48 h post-hatch (hph) for each breeding cohort and mass-spawning night.

		Tank A		Tank B		Tank C	
		Night 1	Night 2	Night 1	Night 2	Night 1	Night 2
Hatch	TL (μm)	NA	1626 ± 38^{c}	1777 ± 18^{b}	1650 ± 17^{c}	1893 ± 46^{b}	2069 ± 23^{a}
	YS (μm)	NA	695 ± 7^{c}	882 ± 6^{a}	901 ± 11^{a}	791 ± 8^{b}	773 ± 11^{bc}
	OG (μm)	NA	321 ± 2^{a}	282 ± 3^{b}	257 ± 2^{c}	247 ± 3^{c}	260 ± 4^{c}
24 hph	TL (μm)	NA	2369 ± 12^{ab}	2431 ± 36^{a}	2440 ± 32^{ab}	2347 ± 16^{b}	2382 ± 26^{ab}
	ED (μm)	NA	230 ± 4^{b}	232 ± 13^{ab}	232 ± 3^{b}	225 ± 3^{b}	248 ± 3^{a}
	OG (μm)	NA	274 ± 4^{bc}	440 ± 15^{a}	303 ± 4^{ab}	265 ± 3^{c}	265 ± 4^{c}
48 hph	TL (μm)	NA	2443 ± 35^{abc}	2306 ± 24^{bc}	2414 ± 21^{ab}	2480 ± 15^{a}	2199 ± 22^{c}
	ED (μm)	NA	224 ± 1^{ab}	234 ± 4^{ab}	223 ± 4^{b}	248 ± 4^{a}	237 ± 4^{ab}
	OG (μm)	NA	146 ± 12^{ab}	246 ± 12^{a}	200 ± 5^{a}	140 ± 3^{b}	138 ± 3^{b}

Data are displayed as mean ± standard error for $n = 20$ except for Tank A (night 2 at 24 hph $n = 10$ and 48 hph $n = 2$). Abbreviation: NA, not applicable; hph, hours post hatch; TL, total length; YS, yolk sac Feret's diameter; ED, eye diameter; OG, oil globule Feret's diameter.

distributed (Fig. 1; Supplementary Table 2).

3.5. Genetic diversity

The broodstock cohort from Tank A showed the highest average number of alleles (3.5/locus), and expected (0.502) and observed (0.536) heterozygosity, followed by the cohorts from Tank B (3.19/locus, 0.488 and 0.504) and Tank C (3.06/locus, 0.481 and 0.543; Table 8). The F_{is} value for the three broodstock cohorts was negative and significantly <0 ($P < 0.05$) for Tank C. Exact tests revealed that all 16 microsatellites conformed to HWE in the broodstock from Tanks B and C. In contrast, one microsatellite deviated from HWE in broodstock from Tank A (Supplementary Table 4). The F_{is} values for offspring across all spawns were negative and significantly <0 ($P < 0.05$; Table 8).

3.6. Effect of male reproductive condition on spawning performance and early embryonic development

Overall, there was a moderate-weak linear relationship between each male's paternal contribution to progeny and their different sperm quality parameters at 2.5 hpf and 24 hph. There was a positive correlation between sperm concentration and paternal contribution 2.5 h after fertilization for Night 1 ($r(22) = 0.45$, $P < 0.05$), but no significant relationships were found between sperm quality parameters and paternal contribution for Night 2 (Table 9). Paternal contribution 24 h after larvae hatched for Night 1 was, however, negatively correlated to milt volume ($r(22) = -0.58$, $P < 0.05$). In contrast, for Night 2, it was negatively correlated to condition factor ($r(22) = -0.44$, $P < 0.05$), VCL ($r(22) = -0.46$, $P < 0.05$), and VAP ($r(22) = -0.43$, $P < 0.05$; Table 9).

PCA conducted using all males showed the first five principal components accounted for 81.8% of the total variance present in the study. Of these, PC1 and PC2 accounted for 33.01% and 17.09%, respectively (Fig. 2a). The first component (PC1) was significantly positively correlated with sperm concentration ($r(22) = 0.77$, $P < 0.0001$), milt volume ($r(22) = 0.64$, $P < 0.01$), paternal contribution to embryos 2.5 hpf for Night 2 ($r(22) = 0.64$, $P < 0.01$), and paternal contribution to larvae 24 hph for the Night 1 ($r(22) = 0.64$, $P < 0.01$). However, PC1 was negatively correlated with VSL ($r(22) = -0.86$, $P < 0.001$), VAP ($r(22) = -0.82$, $P < 0.001$), VCL ($r(22) = -0.78$, $P < 0.001$), condition factor ($r(22) = -0.75$, $P < 0.0001$), sperm DNA damage ($r(22) = -0.73$, $P < 0.001$), and BW ($r(22) = -0.56$, $P < 0.01$) . This result suggests that these ten variables are co-variables, meaning that, in general, smaller males had higher milt volume extracted, higher sperm concentration, reduced sperm velocities and DNA damage, and a higher contribution to the larvae that survived at 24 hph. The second component (PC2) was significantly positively correlated with TL ($r(22) = 0.75$, $P < 0.0001$), paternal contribution to larvae 24 hph for Night 2 ($r(22) = 0.63$, $P < 0.01$), BW ($r(22) = 0.50$, $P < 0.05$), sperm DNA damage ($r(22) = 0.49$, $P < 0.05$), and paternal contribution to embryos 2.5 hpf for Night 2 ($r(22) = 0.48$, $P < 0.05$). However, PC2 was negatively correlated to ALH ($r(22) = -0.69$, $P < 0.001$), TM ($r(22) = -0.50$, $P < 0.01$), and VCL ($r(22) = -0.43$, $P < 0.01$). The second component suggests that larger males generally have a lower proportion of motile spermatozoa with reduced VCL and ALH swimming ability, higher levels of sperm DNA damage, and a higher contribution to the early embryos 2.5 hpf on Night 2. Thus, they might have participated predominantly during the second spawning night. The third component (PC3; 13.1% of total variance) was characterized by a significant positive relationship with sperm viability ($r(22) = 0.83$, $P < 0.0001$), TM ($r(22) = 0.56$, $P < 0.01$), BCF ($r(22) = 0.56$, $P < 0.01$), and milt volume ($r(22) = 0.46$, $P < 0.05$), suggesting that larger milt samples at collection have higher viability and TM and BCF motility characteristics. The fourth component (PC4; 11.2% of total variance) was positively correlated with paternal contribution to embryos 2.5 hpf for Night 2 ($r(22) = 0.58$, $P < 0.01$) and paternal contribution to larvae 24 hph for Night 2 ($r(22) = 0.49$, $P < 0.05$), but

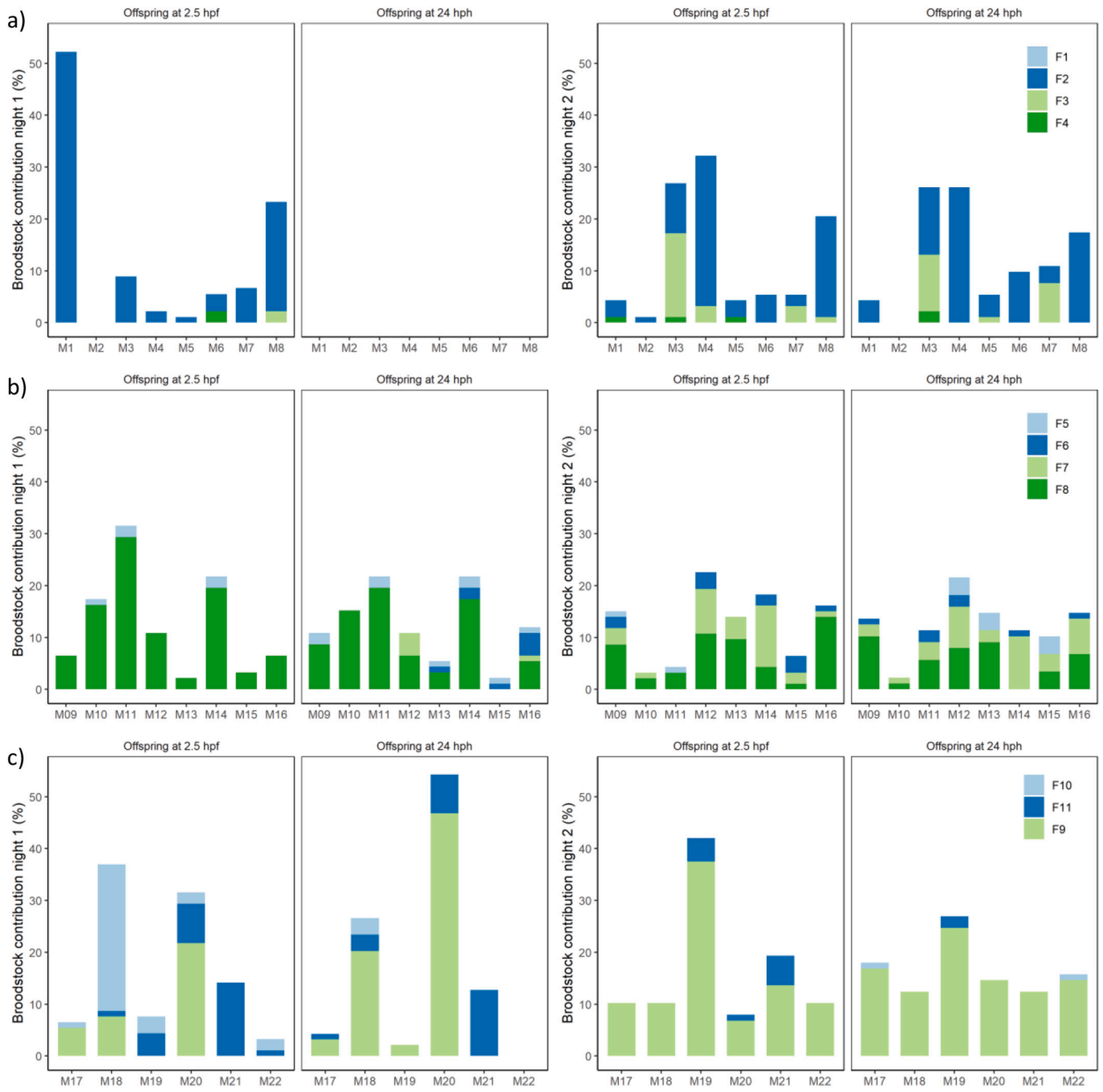

Fig. 1. Proportion of barramundi (*Lates calcarifer*) broodstock contributing to parentage of progeny at 2.5 h post-fertilization (hpf) and 24 h post-hatch (hph) on the first and second night of mass-spawning in (a) Tank A ($n = 12$), (b) Tank B ($n = 12$) and (c) Tank C ($n = 9$). Each male's contribution is displayed on the y-axis, while each female's contribution is shown by the stacked bar plots.

negatively correlated with paternal contribution to embryos 2.5 hpf for Night 1 ($r(22) = -0.66$, $P < 0.001$), TL ($r(22) = -0.56$, $P < 0.05$), and BW ($r(22) = -0.46$, $P < 0.05$). This PC4 suggests that smaller males had a higher contribution to progeny during the spawning on Night 2 compared to Night 1. The fifth component (PC5; 7.4% of total variance) was negatively correlated with BCF ($r(22) = -0.56$, $P < 0.01$) and paternal contribution to larvae 24 hph for Night 1 ($r(22) = -0.55$, $P < 0.01$), implying that males producing spermatozoa with reduced BCF might have a reduced contribution to the larvae that survived to 24 hph. While the primary variables responsible for the variation of each component remain similar across tanks, the dynamic and the weight of each variable differed across tanks (Fig. 2b-d). For example, PCA using individuals from Tank A showed PC1 and PC2 explained 41.76% and 26.28%, Tank B showed 31.09% and 27.28%, and Tank C showed 42.75% and 25.14% of the total variance, respectively (Fig. 2).

4. Discussion

In this study, the use of advanced sperm quality assessment on barramundi broodstock revealed the presence of a high variation in physical traits, milt characteristics, and sperm quality between males. Although variation in sperm quality was high, the correlations between sperm quality parameters and paternal contribution to newly fertilized embryos and 24 hph larvae were moderate for sperm concentration and

Table 8
Measures of genetic diversity of progeny generated from mass-spawning events among different cohorts of barramundi (*Lates calcarifer*) broodstock at 2.5 h post-fertilization (hpf) and 24 h post-hatch (hph) across two consecutive spawning nights.

	Tank A					Tank B					Tank C				
	Broodstock	Offspring				Broodstock	Offspring				Broodstock	Offspring			
	–	Night 1		Night 2		–	Night 1		Night 2		–	Night 1		Night 2	
		2.5 hpf	24.hph	2.5 hpf	24 hph		2.5 hpf	24 hph	2.5 hpf	24 hph		2.5 hpf	24 hph	2.5 hpf	24 hph
N_c	12	91	–	93	93	12	92	92	93	88	9	92	94	88	89
k	3.50	2.44	–	2.81	2.75	3.19	2.56	3.13	3.13	3.13	3.06	3.19	3.13	3.06	2.88
A_r	3.10	2.43	–	2.79	2.75	2.88	2.56	3.10	3.13	3.12	2.93	3.19	3.11	3.06	2.85
H_o	0.536	0.554	–	0.542	0.552	0.504	0.524	0.536	0.557	0.534	0.543	0.537	0.544	0.518	0.542
H_e	0.502	0.42	–	0.442	0.448	0.488	0.413	0.442	0.474	0.465	0.481	0.471	0.466	0.427	0.436
F_{is}	−0.068	−0.319*	–	−0.226*	−0.232*	−0.034	−0.27*	−0.215*	−0.176*	−0.149*	−0.130*	−0.141*	−0.168*	−0.214*	−0.244*
N_e	–	2.052	–	3.407	3.185	–	1.630	4.590	6.560	8.446	–	6.542	3.298	2.202	2.897
ΔF	–	0.244	–	0.147	0.157	–	0.307	0.109	0.076	0.059	–	0.076	0.152	0.227	0.173
N_e/N_c	–	0.015	–	0.009	0.010	–	0.019	0.007	0.005	0.004	–	0.006	0.013	0.019	0.014

* indicate a significant deviation from expected Hardy-Weinberg proportions ($P < 0.001$). Abbreviations: N_c, sample size; k, number of alleles; H_o and H_e, average observed and expected heterozygosity respectively; A_r, allelic richness; F_{is}, average inbreeding coefficient; N_e, effective population size; ΔF, rate of inbreeding.

Table 9
Relationship between reproductive condition on spawning performance and early embryonic development of captive-bred male barramundi (*Lates calcarifer*; n = 22) broodstock.

	BW	TL	K	Vol.	Conc.	TM	PM	Slow	Medium	Fast	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	Live	DNA
2.5 hpf N1	0.03	0.32	−0.34	0.23	**0.45***	0.07	0.00	0.31	0.31	0.02	−0.24	−0.23	−0.2	−0.1	−0.09	−0.03	0.05	−0.18	0.24	−0.31
2.5 hpf N2	0.32	0.00	−0.42	0.35	0.13	−0.09	−0.11	0.05	0.25	−0.17	−0.41	−0.23	−0.39	−0.04	0.03	−0.16	−0.19	0.10	0.03	−0.07
24 hph N1	0.05	0.32	−0.43	**−0.58***	−0.04	0.17	0.17	0.06	0.11	0.19	0.29	0.09	0.31	0.05	−0.01	0.23	0.15	0.07	0.04	−0.38
24 hph N2	−0.17	0.18	**−0.44***	0.24	0.15	−0.26	−0.28	−0.03	0.14	−0.34	**−0.46***	−0.19	**−0.43***	−0.01	0.08	−0.18	−0.27	0.10	−0.01	0.02

Matrix of Pearson correlation coefficients between all variables. The data were logit transformed for body condition, fast motility, sperm viability, and sperm DNA damage, or log10 transformed for VCL, VSL, and VAP. Abbreviation: BW, body weight; TL, total length; K, body condition; Vol., milt volume; Conc., sperm concentration; TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, the amplitude of lateral head displacement; BCF, beat cross frequency; Live, the proportion of viable sperm; DNA, the proportion of sperm DNA damage; 2.5 hpf N1 and N2, 2.5 h post fertilization Night 1 and Night 2; 24 hph N1 and N2, 24 h post hatch Night 1 and Night 2. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ denote level of statistical significance.

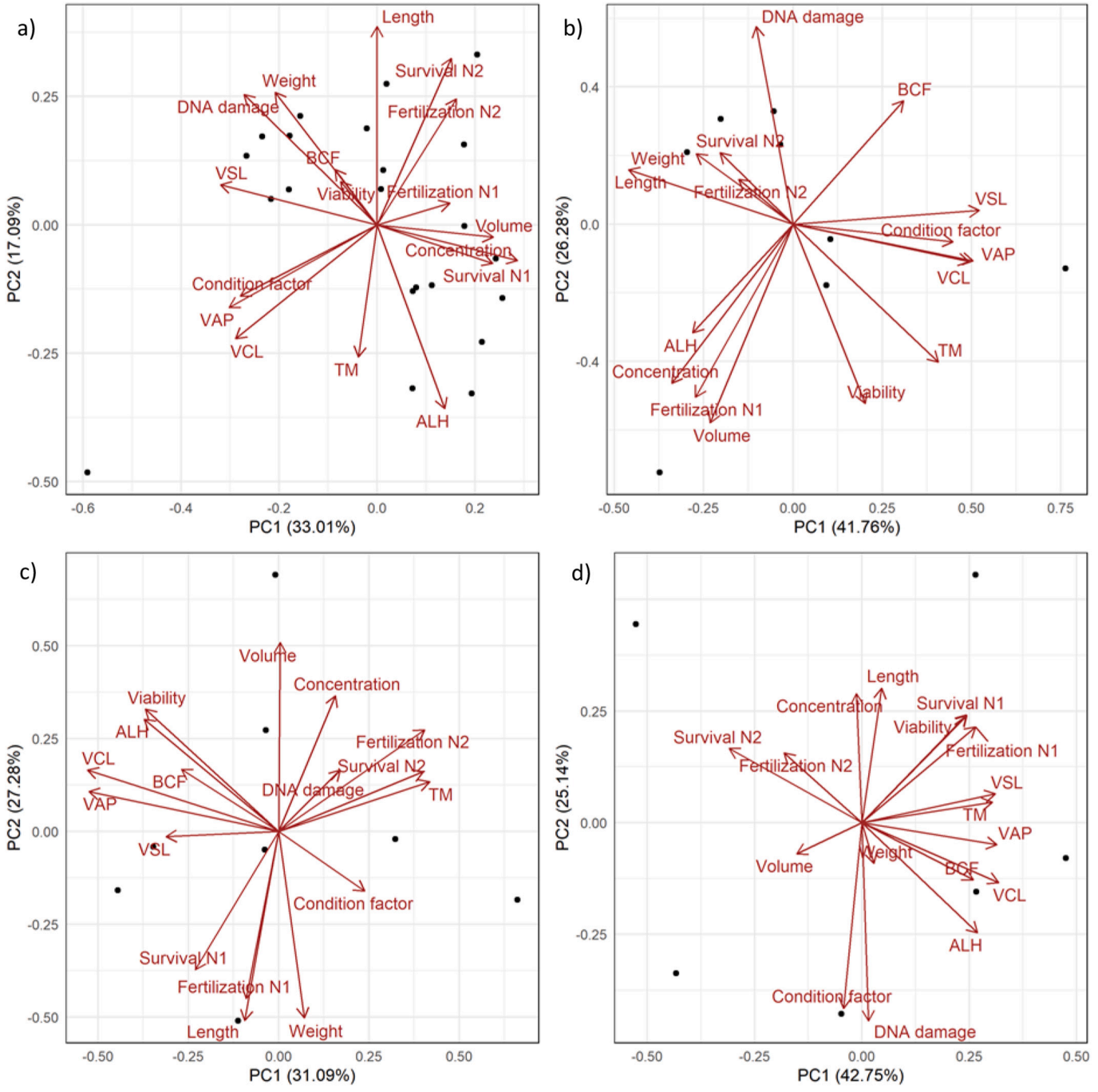

Fig. 2. Principal component analysis (PCA) of male barramundi (*Lates calcarifer*) broodstock reproductive condition and performance during a mass-spawning event over two consecutive nights (N1 and N2). (a) all males $n = 22$, (b) Tank A males $n = 8$, (c) Tank B males $n = 8$, and (d) Tank C males $n = 6$. Dots represent individual males. The proportion of variance captured is given as a percentage for the first and second principal components (PC1 and PC2).

condition factor, respectively. However, for most other sperm quality parameters, the relationships were relatively weak. A reduction in skewed paternity was also found on Night 2 of spawning compared to Night 1. Additionally, males from Tank B with the lowest level of sperm DNA damage resulted in the least skewed parental contribution. While sperm quality could affect the paternity of progeny, it was also found that maternity was highly skewed across all spawning events. Moreover, other unmeasured factors, including artificial spawning induction, spawning behavior, social hierarchy, and tank environment, may have played a role in skewing family size. These data offer the first insight into the dynamics influencing the outcome of mass-spawning events and a baseline for further research into captive breeding of barramundi.

In this study, sperm quality was variable between males but associated with their physical condition. Specifically, males with a higher condition factor had higher sperm motility and reduced sperm concentration. These traits are characteristics of males at an advanced stage of spermatogenesis called spermiation (Schulz et al., 2010). Spermiation is characterized by hydration of the milt in the testes, increasing milt volume, and decreasing sperm concentration (Mylonas et al., 2017; Schulz et al., 2010). During this process in the sperm duct, the increase in seminal plasma allows maturing spermatozoa to acquire motile capability (Schulz et al., 2010). However, males with a higher condition factor also yielded lower milt volume at collection than males with a lower condition factor, producing highly concentrated spermatozoa of

low motility. This divergence is likely due to the cannulation method (Ohta et al., 1997; Suquet et al., 2000). In contrast to the milt volume collected by hand stripping, the milt volume collected by cannula is prone to variation in the time, pressure, and intensity exercised by the technician to extract milt samples during the cannulation of different fish. However, hand stripping is currently not viable on commercial broodstock due to the associated risk of physical injuries. Therefore, milt volume cannot be used as an absolute measure and interpreted as a traditional indicator of reproductive condition in this study.

The presence of sperm DNA damage was also associated with males displaying spermiation characteristics (i.e., higher body weight and condition factor, lower sperm concentration, and spermatozoa with higher velocity and linear swimming trajectory). Males in spermiation have a high proportion of mature spermatozoa, which exclusively rely on antioxidants in seminal plasma to neutralize oxidative compounds (Figueroa et al., 2020). Oxidative compounds, such as reactive oxygen species (ROS), damage sperm DNA (Aitken et al., 2013), which cannot be repaired due to the extrusion of cytoplasmic organelles (Olsen et al., 2005). Moreover, Billard and Takashima (1983) reported that spermatozoa stored for an extended period in the sperm duct started degenerating by breaking down chromatin into filaments. Either condition experienced by males during the rearing process may be generating ROS, affecting sperm DNA integrity, or mature spermatozoa of males in spermiation were in the process of reabsorption within the testes. Further examination of sperm DNA integrity under different rearing conditions and developmental stages is warranted to understand the DNA damage levels observed in this study.

The presence of sperm DNA damage in males was also negatively associated with larval survival rate. Embryo loss during early development has been linked in several studies to sperm DNA damage (Depincé et al., 2020; Figueroa et al., 2020; Pérez-Cerezales et al., 2010) and paternal effects on early life-history traits (Butts et al., 2009; Butts and Litvak, 2007a, 2007b; Rideout et al., 2004; Saillant et al., 2001; Siddique et al., 2017). Sperm DNA damage induces malformation and mortality in early larval development, which has been linked to the location of paternal supporting embryonic growth genes (e.g., insulin growth factor (*Igf*), growth hormone (*Gh*), and insulin (*Ins*)) with the chromatin structure of the genome (Cartón-García et al., 2013; Pérez-Cerezales et al., 2011). Paternal genes supporting embryonic growth are compartmentalized in less compact chromatin arrays that can be readily accessed for early transcription (Figueroa et al., 2020). As a trade-off, genes located in less compact arrays may be more sensitive to oxidative damage, leading to alterations in the transcription of these genes and ultimately to developmental arrest (Figueroa et al., 2020). Although males with higher levels of sperm DNA damage showed lower paternity of 24 hph larvae on the first spawning night, there was no relationship between paternity and sperm DNA damage on the second night. This difference in paternity success between nights might be explained by the purging of DNA-damaged spermatozoa on Night 1, resulting in an increased proportion of spermatozoa with high DNA integrity for spawning on Night 2, thereby facilitating optimal egg fertilization and production of healthy larvae. It is known that, for instance, zebrafish, which have anastomosing tubular testes, have sperm production following a pattern of cystic spermatogenesis over a tightly defined six-day cycle (Reinardy et al., 2013). However, barramundi have lobular testes (Guiguen et al., 1994), following semi-restricted spermatogonial sperm production. While the length of the sperm production cycle for barramundi from early spermatid to the production of mature spermatozoa remains to be determined, the semi-restricted spermatogonial sperm production could allow rapid renewal of mature spermatozoa for the second spawning night. This hypothesis should be explored further to better inform our understanding of sperm quality on spawning success in barramundi.

Assessment of sperm function revealed no positive association between sperm motility and fertilization success. Instead, it was found that broodstock with highly concentrated and low motile spermatozoa had higher paternity during spawning. This outcome is in contrast with most reproductive studies. Sperm motility is usually strongly associated with fertilization success (Beausoleil et al., 2012; Fauvel et al., 1999; Gage et al., 2004; Linhart et al., 2005). This disparity may be attributed to the presence of DNA damage in mature, highly motile spermatozoa, impairing fertilization, and the timing of sperm quality assessment, as sperm quality assessment was performed on males prior to the injection of LHRHa to induce a spawning event. LHRHa injection is commonly used in several species to enhance milt hydration in males of lower sperm quality (Mylonas et al., 2017), which will consequently enhance sperm motility and is used by commercial barramundi hatcheries to improve male contribution during spawning (Schipp et al., 2007). However, in this study, a reduction in levels of skewed paternity was not observed compared to mass-spawning events in which males were not injected in previous studies (Domingos et al., 2014, 2013; Loughnan et al., 2013).

In this study, male broodstock in Tank B had the lowest mean level of sperm DNA damage (mean: 0.07 ± 0.03%) compared to Tank C (mean: 2.64 ± 1.18%), and then Tank A (mean: 15.67 ± 6.85%), resulting in the least skewed paternity. In addition, the skew in paternity appears less on the second than the first spawning night (Fig. 1). That said, M5, M6, and M7, with the highest level of sperm DNA damage (19.3%, 24.7%, and 58.9%, respectively), had very little improvement in paternity success on the second night of spawning (Fig. 1a). The limited improvement in paternity success during Night 2 for these males might be due to the sperm apoptosis process being well underway and the result of sperm DNA damage exceeding the capacity of the egg to DNA repair post-fertilization. For instance, in rainbow trout (*Oncorhynchus mykiss*), it has been determined that eggs can repair about 10% of sperm DNA fragmentation (Pérez-Cerezales et al., 2010). Consequently, characterizing spermatogenesis in barramundi from spermatogonia to mature spermatozoa, as well as investigating factors inducing DNA damage, could improve current commercial hatchery practices, improve sperm integrity, and potentially reduce skewed paternity.

The divergence in parameters driving spawning outcomes between tanks and the presence of highly skewed maternity indicate additional factors, such as stress, tank environment, spawning behavior, and social hierarchy, may influence individual male fertility and paternity success. Stress has been shown to affect male-male hierarchies in Atlantic cod (*Gadus morhua*) and alter courtship sequences during mating aggregations (Morgan et al., 1999; Tuyttens and Macdonald, 2000). The effect of stress on barramundi mating behavior has not been assessed; however, sequential egg releases are commonly observed with an interval ranging from 2 min to 3 h between females or releases (unpublished data). The stress associated with hormone treatment 48 h before spawning (including draining the tank, netting, anesthesia, cannulating the broodstock, and LHRHa injection) may have disturbed broodstock hierarchies and mating behaviors, further contributing to paternal skew. Group hierarchy is a major factor affecting spawning synchronization and skewed paternity in many species (Bekkevold et al., 2002; Coe et al., 2008). Research on zebrafish has also shown that social hierarchy affects individual sperm quality and, ultimately, siring performance (Zajitschek et al., 2017, 2014). Moreover, males' reproductive behavior and social status may be passed on to offspring through sperm-mediated genetic and epigenetic effects of sperm fitness (Zajitschek et al., 2017, 2014). Thus, variation in paternity success among male captive-bred broodstock during mass-spawning events may also be influenced by the complex underlying environmental and social cues experienced by their parents inherited through the paternal line.

Although additional research investigating the causes of skewed paternity and maternity may provide valuable knowledge, these undertakings might not be sufficient on their own to limit the variability in spawning outcomes and gain greater control over the contribution of broodstock to the next generation of offspring. While genetic diversity among broodstock may be sufficiently high, inbreeding coefficients resulting from current spawning practices in our study ranged from 5.9

to 17.3%, far exceeding the recommended average of 0.5% per generation for a population to be used for a selective breeding program (Sonesson et al., 2005). Development of advanced breeding technology for barramundi is in its infancy (Marc, 2021; Marc et al., 2023); however, a shift to artificial fertilization might be necessary to overcome genetic loss associated with mass-spawning strategies used in some selective breeding programs (Domingos et al., 2013; Loughnan et al., 2013; Macbeth and Palmer, 2011; Robinson et al., 2010).

5. Conclusion

This study is the first to explore interactions between male barramundi sperm quality and paternity success during mass-spawning events. Weak correlations between traditional predictors of reproductive success and spawning outcomes were observed. In contrast to the expected results, males with a lower body condition performed better during mass-spawning events. While our data highlight abnormal levels of sperm DNA damage in some captive-bred broodstock and the putative effect this has on skewing paternal genetic contribution to early larval stages, further research is warranted to identify other causative factors to improve breeding practices in barramundi.

CRediT authorship contribution statement

Adrien F. Marc: Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jarrod L. Guppy:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Julie Goldsbury:** Methodology, Investigation, Formal analysis. **Donna Rudd:** Writing – review & editing, Supervision. **Holly S. Cate:** Writing – review & editing, Resources, Funding acquisition. **Damien B.B.P. Paris:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Dean R. Jerry:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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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.2024.740717.

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