Development of advanced reproductive techniques to characterize fertility and accelerate selective breeding in barramundi (*Lates calcarifer*)

Thesis submitted by

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July 2021

In fulfillment of the requirements for Doctorate of Philosophy (Science)

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Publication Records

Peer-reviewed publications from this thesis:

1. Chapter 3: **Marc, A. F.**, Guppy, J. L., Bauer, P., Mulvey, P., Jerry, D. R., and Paris, D. B. B. P. (2021) Validation of advanced tools to evaluate sperm function in barramundi (*Lates calcarifer*). Aquaculture, 531, 735802

Conference presentations from this thesis:

1. **Marc, A. F.**, Guppy, J. L., Jerry, D. R., Mulvey, P., Bauer, P. and Paris, D. B. B. (2019) Evaluating barramundi (*Lates calcarifer*) sperm quality using high throughput advanced reproductive tools. In: [Oral presentation at Aquaculture Europe 2019]. From: Aquaculture Europe 19, 8–10 October 2019, Berlin, Germany.

2. **Marc, A. F.**, Guppy, J. L., Goldsbury, J., Rudd, D., Cate, H. S., Paris, D. B. B. P. and Jerry, D. R. (2021) Sperm quality in captive-bred barramundi (*Lates calcarifer*): Effect on spawning performance and paternal contribution. In: [ePoster presented at Aquaculture Europe 2020]. From: Aquaculture Europe 20, 12–15 April 2021, online conference.

3. **Marc, A. F.**, Guppy, J. L., Marshall, H., Jerry, D. R., Rudd, D.* and Paris, D. B. B. P.* (2021) An optimized non-activating medium for short-term storage of barramundi (*Lates calcarifer*) milt. In: [ePoster presented at Aquaculture Europe 2021]. From: Aquaculture Europe 21, 4–7 October 2021, Funchal, Madeira. *The work was jointly conducted in the laboratories of these two senior authors

Statement of the Contribution of Others

The following table describes the nature of the contribution of co-contributors for this thesis.

Chapter	Title	Nature of contribution of co-contributors
1	General introduction	**Adrien F. Marc:** Conceptualization, Writing – Original Draft **Donna Rudd:** Writing – Review & Editing, Supervision **Dean R. Jerry:** Writing – Review & Editing, Supervision
2	Underlying mechanisms contributing to reproductive performance in captive male broodstock: the significance of sperm quality and broodstock conditioning	**Adrien F. Marc:** Conceptualization, Writing – Original Draft **Damien B.B.P. Paris:** Supervision **Donna Rudd:** Writing – Review & Editing, Supervision **Dean R. Jerry:** Writing – Review & Editing, Supervision
3	Validation of advanced tools to evaluate sperm function in barramundi (*Lates calcarifer*)	**Adrien F. Marc:** Conceptualization, Methodology, Investigation, Project administration, Software, Data curation, Formal analysis, Visualization, Writing – Original Draft, Funding acquisition **Jarrod L. Guppy:** Investigation, Methodology, Writing – Review & Editing, Funding acquisition **Paige Bauer:** Investigation **Peter Mulvey:** Methodology, Formal analysis **Dean R. Jerry:** Conceptualization, Resources, Writing – Review & Editing, Supervision, Funding acquisition **Damien B.B.P. Paris:** Conceptualization, Methodology, Formal analysis, Visualization, Resources, Writing – Review & Editing, Supervision, Funding acquisition
4	Optimization of a non-activating medium for short-term storage of barramundi (*Lates calcarifer*) testicular milt	**Adrien F. Marc:** Conceptualization, Methodology, Investigation, Project administration, Software, Data curation, Formal analysis, Visualization, Writing – Original Draft, Funding acquisition **Jarrod L. Guppy:** Methodology, Investigation, Project administration, Writing – Review & Editing, Funding acquisition **Hayley Marshall:** Methodology, Investigation **Dean R. Jerry:** Resources, Project administration, Writing – Review & Editing, Supervision, Funding acquisition **Donna Rudd:** Resources, Methodology, Writing – Review & Editing, Supervision **Damien B.B.P. Paris:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Funding acquisition
5	Characterization of sperm quality in captive-bred barramundi (*Lates calcarifer*): effect on spawning performance and paternal contribution in mass-spawning events	**Adrien F. Marc:** Conceptualization, Methodology, Investigation, Project administration, Software, Data curation, Formal analysis, Visualization, Writing – Original Draft, Funding acquisition **Jarrod L. Guppy:** Conceptualization, Methodology, Investigation, Project administration, Writing – Review & Editing, Supervision, Funding acquisition **Julie Goldsbury:** Methodology, Investigation, Formal analysis

		Donna Rudd: Writing – Review & Editing, Supervision
		Holly S. Cate: Resources, Writing – Review & Editing, Funding acquisition
		Damien B.B.P Paris: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Funding acquisition
		Dean R. Jerry: Conceptualization, Resources, Methodology, Writing – Review & Editing, Supervision, Funding acquisition
6	General discussion	**Adrien F. Marc:** Conceptualization, Writing – Original Draft
		Donna Rudd: Writing – Review & Editing, Supervision
		Dean R. Jerry: Writing – Review & Editing, Supervision

The following table shows the persons and organizations who have contributed to this thesis.

Nature of assistance	**Contribution**	**Name and Affiliation of co-contribution**
Intellectual support	Mentoring and peer support	**Diana Mendez:** mentor at the Doctoral Cohort Program, Division of Tropical Health and Medicine, James Cook University, Australia
		Carolyn Smith-Keune: lab manager at Molecular Ecology and Evolution Laboratory (MEEL), James Cook University, Australia
Financial support	Stipend	**International Post-graduate Research Scholarship** Australian Government, James Cook University, Australia
		Advance Queensland PhD Industry Scholarship Queensland Government, Australia
	Research funding	**Large Partnership Grant** James Cook University, Australia
	Industry partner	**Mainstream Aquaculture Group Pty Ltd**, Australia
	Conference support	**Higher Degree Research Enhancement Scheme Round 2 2018** College of Public Health, Medical and Veterinary Sciences, James Cook University, Australia
Data collection	Ethics permit	**A2406** James Cook University Animal Welfare and Ethics Committee, Australia
	Animal husbandry and sample collection	**Mainstream Aquaculture Group Pty Ltd**, Australia

Data Storage

All data from which statistical analyses were performed are available at James Cook University electronic data storage through the following permanent links:

Chapter 3: https://cloudstor.aarnet.edu.au/plus/s/goTlL7G8EnC1rL8

Chapter 4: https://cloudstor.aarnet.edu.au/plus/s/7VM6na0zywbwzix

Chapter 5: https://cloudstor.aarnet.edu.au/plus/s/SM0eJHEG3a1boO9

Acknowledgments

The completion of this PhD thesis is the realization of a long dream of mine, resulting from an inexplicable love for aquaria and fish breeding. This could not have happened without the unconditional support of my family. I would like to express my deepest gratitude to my mother and brothers for believing in me and supporting me on my journey to become a scientist, and to my beloved partner Johannes for his continuous support during the day-to-day rollercoaster of my PhD.

This PhD was an erratic journey, filled with failed trials that helped me learn and refine experiments, eventually leading to successful outcomes and key moments of joy and relief. I admire the barramundi's natural ability to never conform to my initial hypothesis, making it an excellent study species. I could not have done this work without the support of my peer Jarrod Guppy, who shared this entire experience. I could not have asked for a better ally. I am grateful for his friendship and look forward to our next research project.

Finally, I would like to thank my supervisors, Associate Professor Damien Paris and Professor Dean Jerry, for developing this cross-disciplinary PhD project and taking me on board to join them on this adventure. I have evolved and learned so much during this time. A special thanks to Prof Dean Jerry for his support and guidance since Day 1, starting with my arrival in Australia and integration into the Aquaculture Genetics Research Group, to the completion of this PhD. His belief in me has encouraged me to succeed and hold myself to a higher standard. Thank you for being an excellent mentor and a great inspiration. I would also like to thank Associate Professor Donna Rudd for stepping up and supporting me throughout my PhD. Donna motivated me incredibly through her cheerful personality and eternal optimism. My sincere thanks to Dr Diana Mendez for her invaluable feedback throughout the writing stage and unwavering encouragement, and to Dr Melissa Crowe and the DTHM Cohort Doctoral Studies Program.

My research would not have been possible without the financial help from the Australian and Queensland Governments. They granted me residency and awarded scholarships (JCU-PRS and Advance QLD scholarship) that covered my tuition fees and provided a living stipend. In addition, I wish to extend my gratitude to my industry partner Mainstream Aquaculture Group Pty Ltd, for their financial contribution and for granting me access to barramundi broodstock and to the Marine and Aquaculture Research Facilities staff for their assistance in overseeing live support of large recirculating aquaculture systems.

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Abstract

Barramundi (*Lates calcarifer*), also called Asian seabass, is a tropical percoid fish important to aquaculture and wild fishery. Throughout the last decade, demand for farmed barramundi has increased globally due to its good consumer attributes, while its robust and euryhaline physiology and its rapid growth rate have made barramundi attractive for farming. In Australia, barramundi aquaculture is one of the fastest-growing industries, with demand for Australian farmed barramundi outstripping current production. Therefore, the Australian barramundi industry is poised to expand its infrastructure and apply innovative farming practices to continually boost production while maintaining high production standards and product quality. One of the most promising innovations in the field is the use of genetically selected stocks to realize the full growth potential of the species in aquaculture. However, while selection programs are underway, the breeding biology of barramundi adds a layer of complexity to the design of programs and uncertainty regarding the contribution of individual broodstock to progeny cohorts.

Currently, barramundi farming seedstock production relies on the conduct of mass-spawning events (i.e., a spawning involving multiple males and females). However, the outcomes of these mass-spawning events often remain unpredictable in terms of larval quantity and quality and the retention of genetic diversity levels present in the broodstock population. Furthermore, genetic testing performed on progeny generated through mass-spawning events have demonstrated highly skewed paternal contribution post-fertilization, resulting in decreased genetic diversity in the offspring. To better understand the potential role of sperm quality on reproductive performance and enable the development of advanced reproductive technologies (ARTs), including cryopreservation and artificial fertilization, the optimization of advanced sperm function assessments for barramundi is required. As a result, the barramundi aquaculture industry and selective breeding programs will achieve a higher level of control over the pairing of broodstock and seedstock production and improve genetic gain.

The studies reported in this PhD thesis address these issues (detailed in Chapter 1) by reviewing current knowledge and technologies used in reproductive fertility of other aquaculture species to select and optimize advanced sperm function assessments for barramundi (detailed in Chapter 2). This review highlights that aquaculture species' complex and diverse physiology, reproductive strategy, and life history made the translation of reproductive technologies complex across species due to the need for species-specific reproductive knowledge and technological optimization. Moreover, despite emerging initiatives to standardize procedures to assess sperm function, the lack of standardized guidelines and practices adds substantial background variability, clouding outcomes from advanced sperm function assessments. Therefore, the research presented in this thesis highlights the need for species-specific validation of advanced sperm function assessments and thorough reporting of the methodology to improve reproducibility and inter-species comparative analyzes. Finally, the use of advanced sperm function assessments in recent studies permitted the demonstration of a strong link between paternal contribution in post-fertilization and larval survival, thereby providing evidence supporting the development of ARTs for barramundi to improve seedstock supply and larval quality. Overall, Chapter 2 demonstrated the importance of developing and validating advanced sperm function assessments for barramundi to investigate the cause of highly skewed paternal contribution and develop fundamental ARTs to overcome current breeding bottlenecks associated with the traditional mass-spawning mating strategy.

In Chapter 3, the optimization and validation of advanced sperm function assessments were carried out for barramundi. Barramundi sperm morphology was characterized to permit the calibration and validation of automated sperm counting and motility detection via computer-assisted sperm analysis (CASA). Several parameters were examined to determine the optimum settings for accurate CASA sperm counting, and compared to manual haemocytometer counts, including the validation of the sperm detection accuracy by CASA: sample dilution (1:1000, $r = 0.87$), the minimum number of fields ($n = 4$, CV = 7.5 %), and the effect of motile *vs.* immotile spermatozoa on automated counting (no effect, $r = 0.99$, $P < 0.001$). Assays for cell viability (Hoechst/propidium iodide; plasma membrane integrity assay) and DNA integrity (FITC/PI; TUNEL DNA fragmentation assay) were also validated for barramundi

spermatozoa by flow cytometry using 70 °C heat-treated controls and a 5-point intact: damaged dilution curve (r = 0.98, P < 0.001), and DNase-treated sperm controls, respectively. Based on these validated assays and optimized conditions, male barramundi broodstock exhibited a baseline sperm concentration of 15.1 ± 3.6 x 10^9 sperm/mL, 52.8 ± 9.6% total motility, 13.1 ± 4.2% progressive motility, 64.2 ± 3.5% viable spermatozoa, and 43.5 ± 6.0% spermatozoa with DNA damage. In this study, the use of CASA was validated through several technical trials to assess sperm concentration and motility reliably. The dual staining procedures, including Hoechst/PI and FITC/PI stainings, were validated using flow cytometry to assess barramundi sperm viability and DNA integrity. Importantly, these assays permitted a rapid and accurate assessment of up to 100,000 sperm per sample, providing a comprehensive semi-automated assessment of barramundi sperm quality usable at a commercial scale. Furthermore, these optimized assessments provided the first quantitative sperm quality data for barramundi. This preliminary sperm quality data indicated high variation between individuals for each parameter assessed and the presence of high rates of plasma membrane and DNA damages. Therefore, further research was required to ensure the accuracy of the methodologies and investigate the cause of plasma membrane and DNA damages.

In Chapter 4, based on the outcome of the first trials, the effect of handling and storage on sperm quality was evaluated to ensure that the accuracy of the advanced sperm function assessments was not compromised. Marine Ringer's solution (MRS) is a common medium used for storing teleost spermatozoa, including barramundi. However, initial trials showed that the use of MRS for storing testicular milt collected from captive-bred barramundi resulted in spermatozoa losing cell membrane integrity and undergoing lysis within 30 min. This outcome suggested that MRS was not osmotically balanced with the physiological requirements of barramundi spermatozoa collected using testicular cannulation. Thus, seminal and blood plasma's ionic and metabolite composition from captive-bred barramundi were characterized to refine the non-activating medium (NAM) composition. In particular, the effect of osmolality, pH, Na^+, and K^+ concentrations on sperm viability and motility were examined to determine the optimum composition of the non-activating medium to increase the longevity of barramundi spermatozoa *in vitro*. This investigation was achieved through iterative adaptation of the

non-activating medium ionic composition. The data showed that increasing the medium osmolality from 260 to 400 mOsm/kg significantly improved sperm viability. Moreover, replacing the buffering agent $NaHCO_3$ with HEPES significantly enhanced sperm motility and velocity. Testicular milt collected from barramundi by cannulation could be stored at 4 °C without significant loss of motility for up to 48 h when diluted with an optimized non-activating medium containing 185 mM NaCl, 5 mM KCl, and 10 mM HEPES at pH 7.4. Furthermore, progressive sperm motility was retained for up to 72 h storage under these conditions. The optimized non-activating medium developed in this study significantly extends the lifespan of barramundi spermatozoa *in vitro*, permitting the development of ARTs for this species.

Finally, in Chapter 5, correlations with paternal broodstock spawning performance and contribution to progeny cohorts were investigated. The fertility of male barramundi (n = 22) from three different breeding cohorts was assessed. Morphometric measurements of broodstock were recorded (i.e., body weight, total length, and condition factor). Milt samples were collected through testicular cannulation, and milt volume, sperm concentration, and sperm total count were determined. Sperm quality assessment was performed, including sperm motility using CASA and sperm integrity (i.e., viability assay and TUNEL DNA fragmentation assay) using flow cytometry. Mass-spawning events were induced using intramuscular injection of luteinizing hormone-releasing analog. Broodstock spawned on two consecutive nights following the injection. Eggs at 2.5 h and 12 h post-fertilization (hpf) and larvae at 24 h and 48 h post-hatch (hph) were collected to assess spawning success and survival. Offspring collected at 2.5 hpf and 24 hph were also genotyped using a microsatellite pedigree panel to determine their parentage and examine the relationship between sperm quality of individual males and offspring survival. Firstly, it was found that male physical condition and sperm quality were highly variable within each breeding cohort. Males with a lower condition factor showed lower sperm motility, whereas males with a higher condition factor showed higher sperm motility and higher levels of sperm DNA damage. Secondly, highly skewed paternal and maternal contributions were observed in all spawns, resulting in loss of genetic diversity and high inbreeding rates in offspring (5.9 to 24.4%). The total number of eggs released and fertilization rate were variable between spawns and cohorts. Offspring mortality occurred

mainly during embryonic development between 2.5 hpf and hatching at 12 hpf, with 25% of fertilized embryos showing arrested development. Mortality of larvae continued over the next 24 h of larval development, with an additional 25% of larvae dying before larval survival rates stabilized across all spawns between 24 and 48 hph (≤ 90.9%). Finally, the analysis of the relationships between male fertility and spawning performance showed that while all males demonstrated fertilization capability, the paternal contribution was dominated by males with a lower-body condition and high sperm concentration in offspring collected at 2.5 hpf. While the paternal contribution of offspring collected at 24 hpf was also associated with males with a lower-body condition, a strong relationship between sperm DNA damage and larval survival ($r(22) = -0.67$, $P < 0.001$) was observed. Yet, this relationship between sperm DNA damage and larval survival rate was only significant in offspring generated during the first spawning night. Overall, sperm DNA damage might be associated with the presence of mature spermatozoa stored for an extended time in the spermatic duct and may not be inherent to male fertility. Due to the complex dynamics occurring during mass-spawning events, the investment in the development of ART for barramundi is justified and strongly recommended as the way forward to gain greater control over individual contribution, overcome current breeding bottlenecks, and enhance seedstock production and quality.

The research presented in this thesis provides the first optimized methodology to perform a comprehensive fertility assessment in male captive-bred barramundi. Furthermore, the implementation of this methodology allowed, for the first time, to investigate barramundi sperm biology and physiology. This information led to significant improvement in barramundi sperm handling and storage procedure. Having a reliable sperm handling and storage procedure optimized for captive-bred broodstock opens new opportunities for barramundi aquaculture research and industry development. Namely, it allows for interstate transport of sperm samples (e.g., remote sperm quality assessment), provides a base and control solution for developing a cryopreservation procedure, and allows sperm samples of several males to be held readily available to develop an artificial fertilization procedure. Finally, the implementation of an optimized fertility assessment methodology allowed, for the first time, to investigate the role sperm quality may play in variation in spawning performance and skewed paternal

contribution to cohorts of progeny in barramundi. This research provided the first evidence for paternal effects in offspring development and early survival in barramundi and highlighted future research directions to enhance broodstock management practices and seedstock production.

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List of Abbreviations

11-KT	11-Ketotestosterone
17,20β-P	17,20β-dihydroxypregn-4-en-3-one
ALH	Amplitude of lateral head displacement
AM	Activating medium
APPs	Acute-phase proteins
A_r	Allelic richness
ARTs	Advanced reproductive technologies
ATP	Adenosine triphosphate
AU$	Australian dollar
BCF	Beat-cross frequency
BSA	Bovine serum albumin
BW	Bodyweight
Ca^{2+}	Calcium
$CaCl_2•2H_2O$	Calcium chloride dihydrate
$CaCO_3$	Calcium carbonate
CASA	Computer-assisted sperm analysis
CHO	Cholesterol
Cl^-	Chlorine
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
Conc.	Concentration
CV	Coefficient of variation
D^+ glucose	Dextrose
DBD-FISH	DNA breakage detection-fluorescence in situ hybridization
ΔF	Inbreeding rate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
E_2	17β-estradiol
ED	Eye diameter
Eif1b	Eukaryotic translation initiation factor 1b
FBS	Fetal bovine serum
F_{is}	Inbreeding coefficient
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FSH	Follicle-Stimulating hormone
gDNA	Genomic DNA
Gh	Growth factor gene
Glu	Glucose
GnRH	Gonadotropin-releasing hormone
Gnrh1,2	GnRH genes
GPR54	G-protein-coupled receptors
GSI	Gonadosomatic index
h	Heritability estimate
hCG	Human chorionic gonadotropin
HCO_3^-	Bicarbonate

H_e	Expected heterozygosity
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_o	Observed heterozygosity
Hox	Homeotic genes
HPA	Hypothalamic-pituitary-adrenal
hpf	Hour post-fertilization
HPG	Hypothalamus-pituitary-gonad
hph	Hour post-hatching
HPT	Hypothalamic-pituitary-thyroid axis
HSP (90, 70, 60, 5)	Heat shock protein
HUFAs	Highly unsaturated fatty acids
HWE	Hardy–Weinberg equilibrium
ICC	Intraclass correlation coefficient
Igf	Insulin growth factor gene
Ins	Insulin gene
k	Mean number of alleles per locus
K^+	Potassium
KCl	Potassium chloride
K_d/K_s	Mean numbers of offspring per dam and sire
KISS	Kisspeptin
Kiss1,2,3	KISS genes
Kissr	KISS receptors
K	Condition factor
LH	Luteinizing hormone
LHRHa	Luteinizing hormone-releasing hormone analog
LIN	Linearity of the curvilinear path
Mg^{2+}	Magnesium
$MgSO_4•7H_2O$	Magnesium sulphate heptahydrate
MRS or MRS260	Marine Ringer's solution at 260 mOsm/kg
MRS400	Marine Ringer's solution adjusted at 400 mOsm/kg
n	Number of
N1 and 2	Negative control stain
Na^+	Sodium
NaCl	Sodium chloride
$NaH_2PO_4•2H_2O$	Sodium dihydrogen phosphate dihydrate
$NaHCO_3$	Sodium bicarbonate
NAM	Non-activating medium
NaOH	Sodium hydroxide
N_c	Sampling size
N_d/N_s	Number of dams and sires
Ne	Effective population size
Ned/Nes	Effective numbers of dams/sires
OG	Oil globule diameter
Osm	Osmolality
P1/P2	Positive control stain
PBS	Phosphate-buffered saline
PCA	Principal component analysis
pCO_2	Partial pressure of carbon dioxide
PES	Polyethersulfone

PFA	Paraformaldehyde
PI	Propidium iodide
PIT	Passive Integrated Transponder
PL	Phospholipids
PM	Progressive motility
PO_4^3	Phosphate
PUFAs	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
r	Coefficient of correlation
RH123	Rhodamine 123
SCD	Sperm chromatin dispersion test
SCSA	Sperm chromatin structure assay
SD	Standard deviation
SEM	Standard error from the mean
Sox2	SRY(sex-determining region Y)-Box 2
SSC	Side scatter
STR	Straightness of the average path
TC	Total count
TG	Triglycerides
TL	Total length
TM	Total motility
TP	Total protein
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
U1 and 2	Unstained control
VAP	Average path velocity
VCL	Curvilinear velocity
V_d/V_s	Variance in the contribution for dams and sires
Vol.	Volume
VSL	Straight line velocity
Wap65	Warm acclimation protein 65
WHO	World Health Organization
WOB	Wobble
YS	Yolk sac diameter
ΔF	Inbreeding rate
ΔΨM	Sperm membrane potential

Glossary

Activating medium	The diluent used to trigger spermatozoa motility
Advanced sperm function assessments	Cellular and molecular analyses to characterize sperm quality
Anadromous	Fish living in saltwater but breeding in freshwater
Artificial fertilization	The process of fertilizing fish eggs in a laboratory by mixing spermatozoa and ova in media that keep spermatozoa motile
Biomarker	A naturally occurring molecule, gene, or characteristic that indicates a normal or abnormal process or condition
Broodstock	Fish raised for breeding purposes
Catadromous	Fish living in freshwater but breeding in saltwater
Chromatin	The nuclear material, consisting of DNA and protein, that makes up chromosomes
Cryopreservation	The process of long-term storage of cells or tissue at -196 °C used for seed production, genetic management of broodstock, and conservation
DNA damage	Injuries to DNA that introduce deviations from its normal and intact structure, which may alter genetic information and induce apoptosis
Embryo	Refers to an offspring from the fertilization of the egg to the hatching stage
Epigenome	Heritable changes in gene expression that do not result from changes in actual gene sequences
Fertility	The ability of broodstock to produce offspring
Genome	Refers to all genetic material of an organism, including both the genes and the noncoding DNA
Genotyping	The process of identifying the genetic makeup of a fish or stock
Gonad	Refers to the male and female reproductive organ; a testis or ovary
JC-1	Mitochondrial membrane potential probe
Larva (pl. "larvae")	An offspring that hatched and relied on their yolk-sac for their nutrition
Life-cycle	The series of changes in the life of an organism, including reproduction
Meiosis	The division process of a germ cell involving two fissions of the nucleus and giving rise to four gametes
Mitosis	Cell division process that gives rise to genetically identical cells in which the total number of chromosomes is maintained
Non-activating medium	Diluent used to maintain spermatozoa inactive
Oocyte	An immature female gamete
Oocyte maturation	The terminal process of oogenesis during which fully grown oocytes initiate meiotic divisions and become fertilizable
Osmolality	The concentration of dissolved particles of chemicals and minerals in 1 L of diluent divided by its molecular weight
Ovulation	The end process of oogenesis, where mature oocytes are released into the oviduct.
Ovum (pl. "ova")	A mature female gamete that a spermatozoon can fertilize
pH	A measure of the concentration of hydrogen ions presents in a body of water, indicating the acidity or alkalinity level of the solution. Measured on a logarithmic scale from 1 to 10; 7 is neutral
Seedstock	New offspring that are past the larval stage for farming or breeding purposes.
Selective breeding	The process of choosing parents with particular characteristics to breed and produce offspring with more desirable characteristics
Sertoli cell	A somatic cell that facilitates the progression of germ cells to spermatozoa via direct contact and by controlling the environment milieu within the seminiferous tubules
Sperm maturation	Refers to the step following spermatogenesis, during which the spermatozoon gains its ability to respond to motility-activating factors
Sperm quality	Defines the ability of a spermatozoon to successfully fertilize an ovum and initiate the development of a healthy embryo
Spermatic duct	Defines the section connecting the testis to the ureter via the genital pore where spermatids undergo spermiogenesis – also called vas deferens
Spermatid	An immature male gamete formed from a spermatocyte and developing into a spermatozoa

Spermatocyte	An immature male gamete formed from a spermatogonium and dividing into a spermatid by meiosis
Spermatogenesis	The process by which a mature spermatozoon develops from an unspecialized germ cell
Spermatogenic cyst	A cluster of spermatogonia enveloped by Sertoli cells
Spermatogonium (pl. "spermatogonia")	An unspecialized germ cell formed that divides by mitosis to form a primary spermatocyte
Spermatozoon (pl. "spermatozoa")	A male gamete that has reached maturity
Spermiation	Refers to the process in the final stage of spermatogenesis during which non-motile spermatids are released into the spermatic duct
Spermiogenesis	Refers to the development of spermatozoa from spermatids by acquiring motility capability
SYBR 14	Membrane-permeant nucleic acid stain
Teleosts	Refers to species from the Teleostei infraclass, a group including about 26,000 species, accounting for 96% of all fish species

Chapter 1. General introduction

1.1 Fish reproduction: a fragile balance

Reproduction is the biological process by which organisms generate offspring either sexually or asexually (Smith, 1968). This biological process is a fundamental concept of life, as each organism results from reproduction (Smith, 1968). Asexual reproduction permits organisms to generate an identical copy of themselves without the fusion of gametes or change in the number of chromosomes, whereas sexual reproduction involves the combination of the genetic material of two-parent organisms through the joining of haploid gametes (Smith, 1968; Smith and Maynard-Smith, 1978). Sexual reproduction is the most common reproduction form used by animals and plants (Otto, 2008). Most teleosts reproduce sexually and exhibit external fertilization, where gametes are released into the environment to be fertilized (Billard, 1986). Teleosts have the largest number of species among vertebrates and inhabit a wide range of aquatic habitats (Smith and Wootton, 2016). This adaptation has been driven, among other factors, by the diversification of reproductive strategies and tactics, enabling successful reproduction, and thus life over 260 million years across all aquatic environments (Bernardi, 2013). While cues triggering reproduction in teleosts are species-specific, reproduction success depends on a fragile balance of endogenous and environmental stimuli. These stimuli allow the release of gonadotrophic hormones by the hypothalamus to trigger the production of sex steroids, which are responsible for synchronizing the maturation of gametes and spawning (Juntti and Fernald, 2016; Zohar et al., 2010). If well-orchestrated, this multi-factorial biological process is triggered, and reproduction occurs once or multiple times throughout the spawning season, generating quality seedstock to replenish populations (Juntti and Fernald, 2016; Rizzo and Bazzoli, 2020). Reproduction is a fundamental event in the life cycle of every organism and is elementary to the survival of the species; disruption of this process over consecutive years can drive a species to extinction (Rizzo and Bazzoli, 2020).

Since industrialization in the 18th century, the anthropogenization of the environment has affected this fragile balance, leading to the emergence of reproductive disorders and failures in many species (Rizzo

and Bazzoli, 2020). The construction of hydroelectric dams, the transformation of riverbanks, and water pollution are, amongst others, known anthropogenic disturbances that affect teleosts' reproductive success (Arantes et al., 2019; Javed et al., 2016; Sundblad and Bergström, 2014). These anthropogenic disturbances interrupt migratory routes, and change river hydrodynamics and water parameters. Furthermore, these environmental changes prevent mature fish from reaching optimal hormonal levels, inhibiting final gamete maturation (Arantes et al., 2019). Ultimately, natural reproduction is rendered impossible for affected species (Arantes et al., 2019; Rizzo and Bazzoli, 2020). A similar phenomenon can occur when species of commercial importance are kept in captivity for commercial production. The lack of hormonal stimuli, often associated with the simplistic or sub-optimal environment they are reared in, prevents the onset of natural spawning (Mylonas et al., 2017, 2010). In this scenario, the induction of exogenous hormonal treatment is necessary to trigger final gamete maturation and initiate spawning (Mylonas et al., 2017, 2010). With the increasing number of species experiencing reproductive failures in the wild and difficulties in producing seedstock in captivity, alternative methods of natural reproduction have emerged to diagnose reproductive disorders and take control over the reproduction process.

1.2 Advanced reproductive technologies

Advanced/assisted reproductive technologies (ARTs) is an umbrella term describing a range of techniques employed to bypass natural reproduction and generate offspring. For example, ARTs include techniques that allow the assessment of gamete quality (e.g., advanced sperm function assessments), short- and long-term preservation of gametes (i.e., chilled storage, cryopreservation, and vitrification), artificial fertilization of gametes, and embryo transfer/freezing. The use of ARTs by clinicians and scientists has been growing with the rise of infertility and reproductive disorders occurring globally in humans (Inhorn and Patrizio, 2014). These technologies are mainly known to assist couples having trouble conceiving a child naturally. However, they are also used to manage conservation programs for endangered species (Herrick, 2019; Lueders and Allen, 2020) and improve the genetic management of closed captive populations, such as in zoos (e.g., cetaceans, lynx, panda; Lueders and Allen, 2020) and

farms (e.g., livestock; Moore and Hasler, 2017), with the overall aim of gaining control over seedstock production and maintaining and/or enhancing genetic diversity of the closed populations.

In aquaculture, ARTs have been adopted by many industries breeding commercially important species, such as carps, tilapias, salmonids, and catfish (Weber and Lee, 2014). These industries employ ARTs to alleviate many issues and bottlenecks that stem from traditional breeding techniques, including skewed parental contribution to offspring, underrepresentation of genetically valuable individuals, and inconsistent breeding outcomes (Cabrita et al., 2010; Gjedrem, 2012a; Gjedrem et al., 2012; Migaud et al., 2013; Suquet et al., 2000). For instance, the implementation of ARTs has allowed the Atlantic salmon industry to distribute and preserve milt from elite lines (i.e., genetically superior stocks), obtain independent control over the availability of gametes required for production, and facilitate controlled pairing of individuals for genetic selection (Gjedrem, 2012b). Moreover, in conjunction with genetic selection, the use of ARTs on Atlantic salmon (*Salmo salar*) facilitated the first family-based breeding program, enabling a 115% higher body weight gain across the first five generations of selection, as well as the development of additional genetic programs targeting other traits of commercial interest (e.g., disease resistance and meat quality; Gjedrem, 2010; FAO, 2014). As a result, the implementation of genetically selected stocks allowed the Atlantic salmon industry to expand and dramatically increase fillet production from 300 tonnes in 1970 to 2,300,000 tonnes in 2014 (FAO, 2014).

The optimization and establishment of ARTs for commercial aquatic species are paramount to sustaining the growth of aquaculture industries to meet the ever-increasing demand for animal protein. The use of ARTs in aquaculture has the potential to improve the reliability and sustainability of seedstock production and to open a plethora of options to improve traits of commercial importance (e.g., growth, disease resistance, and flesh quality) through the implementation of selective breeding programs (Gjedrem et al., 2012; Gjedrem and Robinson, 2010). With many aquaculture species still in the early stages of the domestication process, the genetic improvement potential of these species to enhance aquaculture production remains largely untapped and foreshadows aquaculture's future growth prospects and opportunities (Gjedrem et al., 2012; Gjedrem and Robinson, 2010).

1.3 Barramundi: ideal candidate species for ARTs

1.3.1 Barramundi in the wild

Barramundi (*Lates calcarifer*), also called Asian seabass, is a tropical percoid species widely distributed in the Indo-West Pacific region (Dunstan, 1959; Grey, 1987). Barramundi is a catadromous and protandrous hermaphrodite species. It migrates down rivers to the sea to spawn and matures as male before changing sex to female later in life (Crook et al., 2017; Dunstan, 1959; Jerry, 2013; Moore and Reynold, 1982). Mature fish spawn and fertilize eggs on nights following full and new moons during the wet season from October to March in Australia (Dunstan, 1959). Spawning activities, generally referred to as mass-spawning events, occur at the mouths of estuaries and around rocky headlands, involving the simultaneous release of gametes from several males and females into the water column (Garrett, 1987). After mass-spawning events, mature fish often remain in saltwater environments (Garrett, 1987). Larvae, however, are transported by currents and tides to brackish nursery habitats (e.g., mangroves, floodplain lagoons, and wetlands) before migrating upstream to creeks and freshwater habitats toward the end of the wet season (Crook et al., 2017; Grey, 1987). It takes between 2 to 4 years for the juveniles to sexually mature as males (Moore, 1979). Once sexual maturity has been reached, mature males return to the estuary to aggregate with females toward the end of the dry season, and larger/older males turn into females generally after reaching 80 cm in length (i.e., 5 to 8 years old; Budd, 2020; Moore, 1979; Roberts et al., 2021).

While the life history of barramundi is complex and involves migrating through several types of aquatic habitats throughout its life, it is a robust species that occupies a wide range of geographic locations and has a strong adaptive genetic capacity (i.e., 6 strains and 21 subpopulations identified in Australia; Unmack, 2001). Although barramundi is classified as a ‘least concern’ species in the Red List of Threatened Species (Pal and Morgan, 2019), its reproduction is not spared from the negative effects of anthropogenic disturbances and climate change (Jerry et al., 2014, 2013; Roberts et al., 2021). For instance, although wild-stocks of barramundi are monitored, and commercial and recreational fishing are regulated, the release of hatchery-produced fingerlings in lakes and rivers by governmental

restocking programs is frequently required to support wild-stock recruitment and fisheries (Loneragan et al., 2013). Restocking programs have been implemented for decades in Australia; however, they necessitate complex genetic management to generate fingerlings with the same genetic makeup as the local populations and maintain high genetic diversity in the wild populations (Loughnan et al., 2019; Newton et al., 2010).

1.3.2 Barramundi in captivity

Globally, barramundi aquaculture breeding and rearing practices differ from country to country. In Australia, the life-cycle of barramundi was closed in captivity in the 1980s (Schipp et al., 2007). Barramundi broodstock are maintained in a reproductive state all year round in tightly controlled culture systems. Barramundi broodstock are kept in water at 30 °C, 30 ppt salinity, and exposed to a 16 h light: 8 h dark cycle, mimicking environmental cues at the peak of the breeding season (Schipp et al., 2007). However, despite being exposed to breeding-inducing environmental cues and supplied with quality feed, a hormonal treatment (luteinizing hormone-releasing hormone analog; LHRHa) is usually required to trigger final gamete maturation and induce a spawning event (Schipp et al., 2007). This current breeding method allows for the production of millions of fingerlings; however, several factors, such as larval quantity, quality, and the retention of genetic diversity levels present in the broodstock population, are unpredictable and highly variable (Domingos et al., 2014, 2013; Frost et al., 2006; Jerry, 2013; Loughnan et al., 2013; Robinson and Loughnan, 2014). For instance, the fertilization rate of two consecutive spawning events can dramatically fluctuate using the same broodstock mating group. Moreover, all genetic analyses performed to date on progeny cohorts have shown a highly skewed parental contribution (Frost et al., 2006; Liu et al., 2012; Loughnan et al., 2013; Wang et al., 2008). For example, a single male can sire up to 55% of offspring, while other males contribute as little as 1% (Frost et al., 2006). This highly skewed paternal contribution influences family sizes and reduces the genetic diversity of offspring produced (Domingos et al., 2014, 2013; Loughnan et al., 2013). While highly variable spawning outcomes can make the supply of seedstock difficult at times, highly skewed parental contribution makes restocking and selective breeding programs challenging and onerous, as the

sampling of a large number of offspring is required to correctly assign parentage through genotyping (Domingos et al., 2014; Loughnan et al., 2013; Sonesson, 2005).

Highly variable spawning outcomes and skewed paternal contributions have been reported in other species raised in captivity, including Atlantic cod (*Gadus morhua*; Herlin et al., 2008), jungle perch (*Kuhlia rupestris*; Hoskin et al., 2015), Japanese flounder (*Paralichthys olivaceus*; Sekino et al., 2003), common snook (*Centropomus undecimalis*; Rhody et al., 2014), and gilthead seabream (*Sparus aurata*; Borrell et al., 2011). For some species, the skewed parental contribution was primarily driven by inconsistent behavioral involvement of male broodstock during spawning events (Sekino et al., 2003; Rhody et al., 2014; Borrell et al., 2011). Lack of behavioral involvement can come from reproductive disorders, including natural inter-individual disparity in sex steroids levels, affecting reproductive aptitude (Cabrita et al., 2006; Chauvigne et al., 2017), suboptimal broodstock rearing conditions, preventing optimal hormonal expression levels, and affecting reproductive behavior and spermatogenesis (Guzmán et al., 2009; Vandeputte et al., 2009), as well as a loss of reproductive traits resulting from domestication (Forne et al., 2009; Morais et al., 2016; Tvedt et al., 2001; Vandeputte et al., 2009). While skewed parental contributions are rife in natural populations due to pre-mating competition (e.g., courting, individual ranking in the group/harem) and post-mating competition (e.g., sperm competition, cryptic female choice), culture conditions and hatchery practices have been linked to increasing this phenomenon (Beirão et al., 2019). Parental contributions of wild barramundi have never been assessed during natural mass-spawning events. However, mass-spawning events performed using wild-caught barramundi broodstock showed a more even contribution amongst individuals compared to captive-bred broodstock (Wang et al., 2008). Research investigating the effect of current husbandry and breeding practices on barramundi broodstock reproduction could help to identify methodology gaps and factors inducing highly variable spawning outcomes and skewed paternal contributions. Moreover, the development of ARTs for barramundi could circumvent these breeding issues by taking control of the breeding process and the genetic makeup transmitted to the next generation (Loughnan et al., 2013; Robinson et al., 2010; Robinson and Jerry, 2009).

1.4 Captive-bred male barramundi: reproductive disorders?

Sourcing new broodstock is a delicate task, as selected individuals will provide and shape the future genetic makeup of the domestic population (Lueders and Allen, 2020). In captivity, the selection of new male barramundi to become broodstock is currently based on a simple health assessment assuming that fertility is consistent with the individual's overall health status (Schipp et al., 2007). Once these new males are identified as potential broodstock, they are introduced to the breeding cohort. Then, before participating in a mass spawning event, sperm motility is visually assessed to ensure male broodstock demonstrate active spermatogenesis and are suitable to be included in the spawning cohort (Schipp et al., 2007). Little is known about how well these practices correlate with fertility and spawning success in barramundi. However, it is clear from studies that additional measures of sperm quality, including viability and DNA integrity, along with accurate measurements of motility, are required to identify biomarkers of fertility in barramundi (Herráez et al., 2017; Robles et al., 2017).

Sperm quality parameters (i.e., sperm motility) have been positively correlated with fertilization success in numerous teleosts (Fauvel et al., 1999; Gage et al., 2004; Rurangwa et al., 2004). Failure of embryonic and larval development has been reported in several teleosts of commercial importance and attributed to a deficiency in spermatozoa and egg quality (Herráez et al., 2017; Rideout et al., 2004a). Investigations of DNA integrity in many teleosts, including zebrafish (*Danio rerio*; Gosálvez et al., 2014), European sea bass (*Dicentrarchus labrax*; Zilli et al., 2003), rainbow trout (*Oncorhynchus mykiss*; Cabrita et al., 2005; Pérez-Cerezales et al., 2009), gilthead seabream (Cabrita et al., 2005), and salmonids (Salmonidae; Dziewulska et al., 2011), demonstrated sperm DNA damage (e.g., nicks and strand breaks) impaired reproductive success and compromised early larval development. Sperm DNA damage occurs in part from the expression of reactive oxygen species resulting from various oxidative stressors, such as xenobiotic substances (Kime, 1995; Kime and Nash, 1999), environmental conditions (e.g., heat stress; Paul et al., 2008; Penã et al., 2017), and inappropriate broodstock management conditions (e.g., poor water quality and excessive handling procedure; De Souza et al., 2015). Research into the factors that induce sperm DNA damage and its downstream effects on reproductive performance

is limited in many species and unknown in barramundi. However, it could be a potential factor causing highly variable spawning outcomes and skewed paternal contributions in hatcheries. Therefore, it is crucial to support the development of ARTs (i.e., advanced sperm function assessments) to diagnose male barramundi reproductive conditions in captivity.

1.5 ARTs: translation from wild to captive-bred barramundi

Previous studies have demonstrated success with collecting and maintaining live spermatozoa of wild barramundi in chilled storage and optimizing cryopreservation using a simple visual estimate of sperm motility (Leung, 1987; Palmer et al., 1993). However, when these published procedures were applied to collect and store milt from captive-bred broodstock in our laboratory, spermatozoa rapidly lost cell membrane integrity and were non-viable within 30 min (Appendix A).

Improper handling and storage procedures can negatively alter sperm functionality and fertilization potential upon activation (Alavi et al., 2004; Bozkurt et al., 2008; Dreanno et al., 1999a; Jing et al., 2009; Yang et al., 2006). In most teleosts, Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} are the main mineral compounds in seminal plasma and, at species-specific concentrations, maintain sperm integrity and fertilization capability (Alavi and Cosson, 2006; Krasznai et al., 2000). Therefore, the biochemical composition of the non-activating medium (NAM) used by Palmer et al. (1993) might have mimicked the seminal plasma characteristic of wild barramundi. However, the rapid loss of plasma membrane integrity in spermatozoa exposed to this NAM indicated a potential change in seminal plasma ionic profile in captive-bred barramundi (see review Alavi and Cosson, 2006). Based on these preliminary results, the methodology used to collect spermatozoa from wild barramundi required substantial optimization to satisfy the biological requirements of spermatozoa collected from captive-bred individuals and allow optimization of ARTs.

1.6 The need for advanced sperm function assessments for barramundi aquaculture

Based on studies conducted on other teleosts, highly variable spawning outcomes and skewed paternal contributions are associated with several factors, including male reproductive capability (Cabrita et al., 2006; Chauvigne et al., 2017; Sekino et al., 2003), the presence of sperm DNA damage (Herráez et al., 2017; Robles et al., 2017), and suboptimal husbandry practices (Guzmán et al., 2009; Vandeputte et al., 2009). Moreover, reproductive procedures performed on wild individuals, such as sperm handling and chilled storage (Leung, 1987; Palmer et al., 1993), could not be transferred without optimization to captive-bred barramundi. The extent to which the hatchery rearing environment influences the male reproductive system, paternal contribution during spawning events, and sperm quality are unknown in barramundi. These issues prevent the implementation of ARTs and taking control of the reproduction process of captive-bred barramundi, which is a major impediment in the effective implementation of restocking and selective breeding programs (Jerry, 2013). Therefore, the optimization of advanced sperm function assessments of captive-bred barramundi is needed to investigate the causes of these issues.

The optimization of advanced sperm function assessments of captive-bred barramundi will enable, for the first time, the comprehensive evaluation of male broodstock reproductive capability, including broodstock morphometries (i.e., body weight, total length, and condition factor), milt characterization (i.e., milt volume, sperm concentration, and total count), sperm motility, sperm morphology, and sperm integrity (i.e., viability and DNA damage). Specifically, the outcome of these assessments will increase our understanding of barramundi reproduction, the current sperm quality status of captive-bred broodstock, and the cause of skewed paternity. These optimized advanced sperm function assessments will also provide a data-driven decision-making process to hatchery operators to identify and exclude sub-fertile individuals from the broodstock cohort, leaving only highly fertile and fecund males in the mating population. Moreover, these assessments will permit quantification of the effect of routine handling procedures and rearing conditions, enabling measured improvements in husbandry procedures.

Advanced sperm function assessments will also permit the optimization of existing ART procedures, such as cryopreservation and artificial fertilization, and make them applicable on a commercial scale for barramundi aquaculture. These procedures will allow control over the breeding procedure, the number of offspring produced, and their genetic makeup and enhance the reliability and quality of seedstock production. The implementation of sperm cryopreservation will facilitate the establishment of gene banks (i.e., enabling long-term preservation of genetic materials), the exchange of disease-free stocks between breeding facilities, and access to an extensive range of genetic profiles. Ultimately, these reproductive techniques will improve the level of control of production quality, providing the ability to breed valuable lines, propagate genetically improved individuals for aquaculture purposes, and offer practical tools to enhance the genetic management of restocking programs. The benefits of developing ARTs and implementing these technologies are paramount and can bring substantial economic growth and competitive advantages for the Australian barramundi aquaculture industry.

Supporting the development of ARTs is particularly relevant for the next decade, as barramundi is a tropical percoid of commercial importance in high demand in domestic and international markets. The barramundi aquaculture industry is currently valued at AU$90 million in Australia, with an annual production of 9,000 tonnes in 2018 – 2019 (FRDC, 2020). While the barramundi aquaculture industry is well established in Australia and has shown strong annual growth (i.e., ~14%), domestic demand for farmed fish outstrips production necessitating the importation from Southeast Asian countries. Currently, imported products represent up to 70% of barramundi sold in Australia annually (FRDC, 2020). Furthermore, with extensive infrastructure investment underway and genetic improvement programs instigated, the industry anticipates reaching an annual production of 25,000 tonnes by 2025 (FRDC, 2020). Therefore, developing and optimizing advanced sperm function assessments and existing ARTs for captive-bred barramundi is a priority to support the expansion of the industry.

1.7 Research aims

The research reported in this thesis had the overarching aim to investigate how sperm quality differs among captive-bred male barramundi broodstock and if sperm quality correlates with (i) egg fertility and (ii) the contribution of males to progeny cohorts, resulting from mass-spawning events. It addresses this aim through four chapters.

Chapter 2: Review of current knowledge on male reproduction mechanisms and advanced sperm function assessments in teleosts.

Chapter 3: Validation of advanced tools to evaluate sperm function in barramundi.

Chapter 4: Optimization of a non-activating medium for short-term storage of barramundi testicular milt.

Chapter 5: Quantify variation in barramundi broodstock sperm quality and its effect on paternal contribution during mass-spawning.

This research was aimed at improving the efficiency and productivity of the barramundi industry by providing a greater understanding of factors that affect male broodstock contribution during mass-spawning events; and the foundation for the development of advanced reproductive technologies with the optimization of sperm handling and short-term storage methodologies for captive-bred barramundi.

Chapter 2. Underlying mechanisms contributing to reproductive performance in captive male broodstock: the significance of sperm quality and broodstock conditioning

2.1 Introduction

In response to challenges associated with supplying the ever-growing global demand for high-quality seedstock (FAO, 2018), sperm quality has received increased attention throughout the aquaculture industry. However, despite significant advances in the last two decades in regards to "closing" the life-cycle of commercially important teleosts and optimizing species-specific larval rearing protocols, numerous bottlenecks remain to efficient reproduction of many aquaculture species (Migaud et al., 2013; Mylonas et al., 2010; Palma and Viegas, 2020). Several factors, including limited spontaneous spawning events (Rasines et al., 2012; Rhody et al., 2014), poor egg fertilization rate (Rhody et al., 2014), arrested embryonic development, and abnormal larval morphology (Nissling et al., 2006), hamper the effective production of seedstock in aquaculture hatcheries. In addition, in some cases, such as in the Senegalese sole (*Solea senegalensis*), a general decrease in sperm and ova production has been observed in captive-bred individuals (Rasines et al., 2012), further reducing the ability to meet the growing demand for high-quality seedstock.

In the wild, a complex set of environmental cues, social interactions, and food availability triggers teleosts' critical reproductive processes (i.e., maturation and spawning; Cardinaletti et al., 2010; Zohar et al., 2010). Each species require an intricate combination of these factors to ensure optimal sexual reproduction and maximize the production of healthy progeny. However, fish are held under intensive production conditions in aquaculture, often within a simplistic environment. Under these conditions, critical reproductive cues are often absent, inhibiting the optimal operation of biological pathways responsible for gametogenesis (Cardinaletti et al., 2010; Rhody et al., 2014). Photoperiod and rearing

temperature are the two main environmental factors manipulated to simulate breeding conditions in the captive environment; however, these are often insufficient to trigger final gamete maturation and spawning events. Therefore, it is common to use hormonal treatment to artificially induce final gamete maturation and spawning behaviors (Loughnan et al., 2013; Migaud et al., 2013; Mylonas et al., 2017).

While the process of oogenesis and factors affecting egg quality have been extensively studied (see reviews Brooks et al., 1997; Kjorsvik et al., 1990; Lubzens et al., 2010), the process of spermatogenesis and male fertility have been overlooked. Indeed, spermatozoa were traditionally considered simple carriers of the male genetic material (Herráez et al., 2017). However, new insights into genetic, epigenetic, and transcriptomic highlighted the critical role of spermatozoa during embryonic development and survival (Herráez et al., 2017; Siddique et al., 2017). Non-genomic information carried by the spermatozoon, in addition to nuclear DNA, has been suggested to drive early embryogenesis and key stages of larval development (Herráez et al., 2017; Robles et al., 2017). Furthermore, paternal DNA and non-genomic materials (e.g., epigenetic modifications and proteins) are sensitive to environmental stressors and contaminants, reducing DNA integrity and altering paternal inherited epigenetic modifications (Herráez et al., 2017). Moreover, biochemical agents produced under environmental or physical stress, such as reactive oxygen species, have also been directly associated with a decline in sperm motility, an increase in DNA damage, and a subsequent decrease in hatching and larval survival rates (Ciereszko et al., 2005; Dietrich et al., 2005; Zhou et al., 2006).

Poor quality spermatozoa are characterized by low motility, abnormal morphology, and a high degree of DNA damage (WHO, 2010). These characteristics are rarely measured in hatcheries, limiting their assessments to visual sperm color and motility measurements. Consequently, poor quality spermatozoa are often undiagnosed, which can be at the origin of poor reproductive performance observed in some species in captivity. Many terrestrial production systems recognized the need to accurately determine and maintain high-quality spermatozoa and have embedded sperm screening into routine breeding practices (Holt et al., 2007). In aquaculture systems, identifying broodstock with poor sperm quality before a spawning event could be key to decreasing the incidence of arrested embryonic development,

improving hatching and larval survival rates, and promoting higher progeny quality (Figueroa et al., 2020; Gosálvez et al., 2014; Herráez et al., 2017; Pérez-Cerezales et al., 2010; Santos et al., 2013). Moreover, high-quality spermatozoa are prerequisites for advanced reproductive technologies (ARTs), including cryopreservation and artificial fertilization, which have proved critical to selective breeding programs in many terrestrial and aquaculture farmed species (Kumar and Engle, 2016; Mylonas et al., 2017, 2010).

Unlike many terrestrial farming systems, where only six primary species (i.e., bovine, porcine, ovine, caprine, equine, and poultry) are farmed, the aquaculture industry cultures a diverse range of species with varied reproductive physiology and requirements. Specifically, 362 species (including hybrids) are farmed around the world (FAO, 2016). While ARTs have been developed for some species, the diversity of habitat/conditions teleosts inhabit and the variety of reproductive strategies employed by teleosts, amongst others, have greatly complicated the transfer of advanced reproductive knowledge and techniques between species (Migaud et al., 2013; Mylonas et al., 2010).

Increased knowledge of the underlying mechanisms affecting spermatogenesis and spermatozoa integrity is a fundamental requirement for optimizing broodstock management and husbandry techniques. This review summarizes the diverse reproductive systems employed by male teleosts while synthesizing current studies that have identified factors causing impaired fertility. Particular attention is given to those studies exploring spermatogenesis and decreased sperm quality in species used in aquaculture. Furthermore, common male broodstock assessment techniques are discussed, and methods for improving standard reproductive assessment are suggested. Finally, this review discusses the importance of sperm quality and its cascade effect on the production of offspring in aquaculture.

2.2 The reproductive biology of male teleosts

2.2.1 An overview of spermatogenesis

Spermatogenesis is the process by which males produce highly specialized haploid cells called spermatozoa. Their role is to transfer the paternal genetic information to an ovum to activate embryonic development. Spermatogenesis occurs in the seminiferous tubules of the testis, and produces spermatozoa from primary stem cells, also called primary undifferentiated spermatogonia. This process is relatively conserved across vertebrates and has been subject to extensive research in teleosts (see reviews Billard, 1986; Schulz et al., 2010).

In brief, spermatogenesis involves multiple stages of morphological and physiological alterations to the developing spermatogonia (Figure 2.1). The primary undifferentiated spermatogonia undergo extensive cellular division, a process called mitosis, during the proliferation phase and give rise to secondary spermatogonia before becoming primary spermatocytes (Figure 2.1). Primary spermatocytes enter the maturation phase, undergo cellular division through meiosis I and II, and give rise to spermatids (Figure 2.1). Spermatids enter the differentiation phase called spermiogenesis and undergo metamorphosis (i.e., develop a flagellum, mitochondria, and in some cases an acrosome) before migrating to the spermatic duct for final maturation, a process called spermiation, to acquire motility capability (Figure 2.1).

While spermatogenesis is a specialized and conserved process, the complexity of the process, involving several morphological and physiological alterations, makes it liable to errors and failures (Herráez et al., 2017). While an estimated 380 mitotic divisions occur during spermatogenesis, only 23 mitotic divisions occur during oogenesis (Vogel and Rathenberg, 1975). Therefore, the likelihood of germline genetic mutations occurring during the generation of a spermatozoon is high compared to oocytes (Fraga et al., 1991) and could be the root cause of fertility issues faced by some aquaculture species.

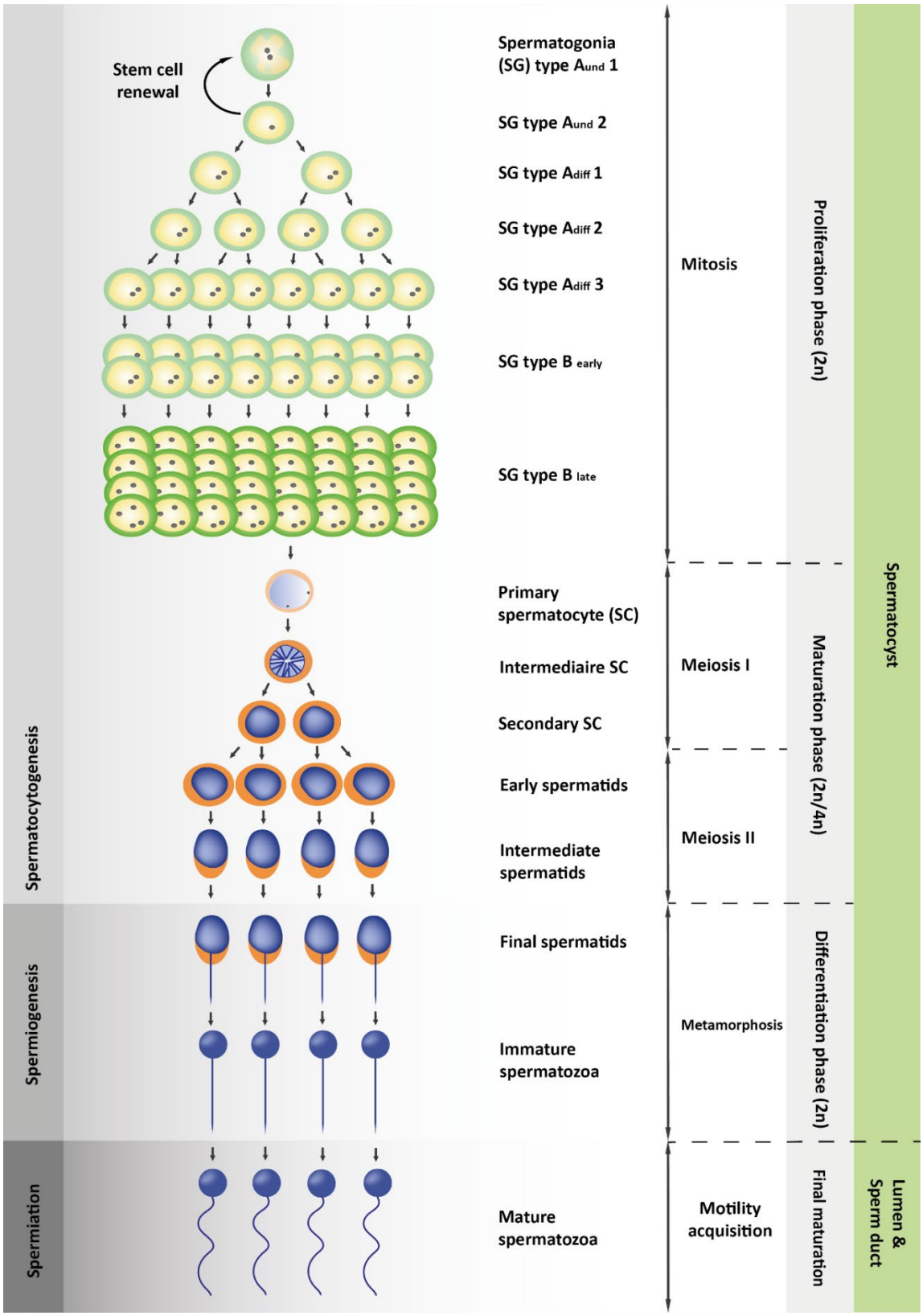

Figure 2.1 Diagram of fish spermatogenesis occurring in the seminiferous tubules of the testis. The process includes three distinct development stages: spermatocytogenesis, spermiogenesis, and spermiation. Abbreviations: spermatogonia (SG), spermatocyte (SC), diploid (2n), haploid (n), chromosome duplication (2n/4n).

The diversification of the testicular structure of teleosts is an additional factor to consider as a source of variability in the interpretation of the outcome of reproductive studies. Specifically, the structure of teleost testis can be divided into two categories based on the morphology of the germinal compartment, which is either known as an anastomosing tubular or a lobular testis (Table 2.1; Parenti and Grier, 2004). In the anastomosing tubular testis, the germinal compartments are a network of interconnected tubules, where spermatogenic cysts, which are a cluster of spermatogonia enveloped by Sertoli cells, form and mature along the entire tubule in an unrestricted manner (Uribe et al., 2014). The anastomosing tubular testis type is mainly present in basal teleosts (e.g., Cypriniformes, Characiformes, and Salmoniformes; Table 2.1; de Siqueira-Silva et al., 2019; Knapp and Carlisle, 2011). In the lobular testis, the germinal compartments are formed of seminiferous lobules with a radial arrangement that connects blindly to the lobular lumen (Uribe et al., 2014). The distribution of the spermatogenic cysts in the lobule can be divided into three types, namely restricted, semi-restricted, and unrestricted, based on the location where spermatogenic cysts form in the lobule (i.e., at the end, at the end and along, and exclusively along the lobule, respectively). The lobular testis type is characteristic of derived teleosts including, amongst others, species from the Perciformes, Atheriniformes, and Beloniformes (Table 2.1; de Siqueira-Silva et al., 2019; Knapp and Carlisle, 2011). While the different phases of spermatogenesis occurring within a spermatogenic cyst remain conserved and rely on hormonal stimuli driven by environmental cues to initiate spermatogenesis and subsequent spawning activities, the progression of developing spermatogenic cysts through the testis structure differs (Cardinaletti et al., 2010; Schulz et al., 2010). These different testis structures among teleosts may affect how spermatozoa are produced and released. Therefore, the testicular structure of teleosts should be considered in the development and customization of ARTs for new aquaculture species as ARTs have mainly been developed for temperate teleosts, which predominantly exhibit anastomosing tubular testes.

Table 2.1 Description of testis type reported in basal and derived teleosts.

Order/ Testis type	Characteristics	Species order	Species	Reference
Basal teleosts				
Anastomosing tubular unrestricted	Spermatogenic cysts form along the tubule and do not migrate from their initial location in the tubule.	Cypriniformes	Common carp (*Cyprinus carpio*)	Gimeno et al. (1998)
		Characiformes	Piranha (*Serrasalmus spilopleura*)	Nóbrega and Quagio-Grassiotto (2007)
		Salmoniformes	Atlantic salmon (*Salmo salar*)	O'Halloran and Idler (1970)
		Gasterosteiformes	Pipefishes (*Syngnathus abaster* and *S. acus*)	Carcupino et al. (1999)
Derived teleosts				
Lobular restricted	Spermatogenic cysts form exclusively at the end of the lobule and migrate toward the spermatic duct, where spermatozoa are released.	Atheriniformes	Rainbowfish (*Melanotaenia fluviatilis)*	Bhatia et al. (2014)
		Cyprinodontiformes	Guppies (*Poecilia reticulate)*	Billard (1984)
		Beloniformes	Medaka (*Oryzias latipes)*	Grier (1976)
Lobular semi-restricted	Spermatogenic cysts form at the end and along the entire lobule. New spermatogenic cysts generated at the end of the lobule create a passive migration of the older spermacyts toward the spermatic duct by appositional growth.	Perciformes	Nile tilapia (*Oreochromis niloticus*)	Vilela et al. (2004)
		Pleuronectiformes	Senegalese sole (*Solea senegalensis*)	García-López et al. (2006)
		Gadiformes	Atlantic cod (*Gadus morhua*)	Almeida et al. (2008)
Lobular unrestricted	Spermatogenic cysts form along the entire lobule and do not migrate during their development.	Synbranchiformes	Marbled swamp eel (*Synbranchus marmoratus*)	Lo Nostro et al. (2003)
		Perciformes	Chanchita (*Cichlasoma dimerus*)	Vázquez et al. (2012)
		Perciformes	Meager (*Argyrosomus regius*)	Prista et al. (2014)

2.2.2 Regulation of spermatogenesis and spawning activities

The regulation of spermatogenesis and spawning activities is tightly conditioned by a complex array of fluctuating environmental cues (Kime and Nash, 1999). For most teleost species, the daily and seasonal fluctuations of environmental cues, including photoperiod, temperature, rainfall, salinity, food availability, and lunar and tidal cycles, are extrinsic factors regulating biological mechanisms, such as spermatogenesis and spawning activities (Bromage et al., 2001). The intensity of the annual variation of an environmental cue often dictates its degree of influence over the biological response for any given species. For instance, photoperiod strongly influences reproductive development and synchronicity for temperate species, while the temperature is a more prominent cue for tropical species (De Vlaming, 1972). The difference in sensitivity to one or multiple environmental cues varies between species and results from the adaptation of teleosts to the environment they inhabit (Table 2.2; Migaud et al., 2013; Wang et al., 2010). The lack of species-specific knowledge about environmental cues that drive reproduction in teleosts is often the first challenge to overcome for successfully controlled propagation. However, while the manipulation of basic extrinsic factors, such as photoperiod and temperature, often suffice to trigger the initiation of gametogenesis for most species in captivity, artificial hormonal manipulation is usually required to trigger final sperm and ovum maturation as well as the synchronous release of gametes during the spawning event (Mylonas and Zohar, 2001). This requirement for an exogenous hormonal treatment to trigger breeding in captivity in several species is due to the oversimplified environmental conditions in which broodstock are maintained all year round (Migaud et al., 2013; Mylonas et al., 2011, 2010). In many cases, the complexity of life-cycle/history (i.e., spawning migration, riverine systems) of species reared in captivity is not, or cannot, be recreated to allow natural gamete maturation and spawning activities (Mylonas et al., 2010).

Table 2.2 Environmental cues that initiate reproductive stimuli according to the geographic residence of the teleost species.

Geographic residence	Environmental cues	Reproductive stimuli	Spawning season	Species	Reference
Higher latitude temperate zones	Photoperiod	Stimulated by increasing day-length.	Summer to late autumn	Atlantic salmon (*Salmo salar*)	Taranger et al. (1999)
				Rainbow trout (*Oncorhynchus mykiss*)	Duston and Bromage (1986)
				Atlantic halibut (*Hippoglossus hippoglossus*)	Norberg et al. (2001)
		Decreasing day-length.	Winter and late spring	Atlantic cod (*Gadus morhua*)	Norberg et al. (2004)
				Haddock (*Melanogrammus aeglefinus*)	Martin-Robichaud and Berlinsky (2004)
Mid to lower temperate latitude	Photoperiod Temperature	Decreasing day-length stimuli upon simultaneous reduction in temperature.		Eurasian perch (*Perca fluviatilis*)	Migaud et al. (2006)
				European sea bass (*Dicentrarchus labrax*)	Carrillo et al. (2010) Felip et al. (2008)
				Striped bass (*Morone axatilis*)	Clark et al. (2005)
				Yellow perch (*Perca flavescens*)	Shewmon et al. (2007)
				Gilthead seabream (*Sparus aurata*)	Kissil et al. (2001)
				Turbot (*Psetta maxima*)	Imsland et al. (2003)
Tropical area	Temperature Photoperiod	Influenced by seasonal differences in temperature.	Spring	Barramundi (*Lates calcarifer*)	Athauda et al. (2012) Athauda and Anderson (2005)
		Photoperiod acting as a secondary cue.	Varied – ephemerides cycle	Nile tilapia (*Oreochromis niloticus*)	Schofield et al. (2011)
Large geographic distribution		Lowest specificity to single environmental cues.	All year round	Common carp (*Cyprinus carpio*)	Sivakumaran et al. (2003)

2.2.3 Pathways of interaction between environmental cues and spermatogenesis

Fish are exposed to daily and seasonal environmental cycles utilized to synchronize reproduction events within species (Wang et al., 2010). Environmental cues such as light are received by photoreceptor organs, such as the retina, the pineal gland, and the encephalic deep brain (Migaud et al., 2010). These photoreceptor organs mediate changes of the light perceived by synthesizing melatonin (Migaud et al., 2010). Amongst other functions, melatonin adjusts the organism's endogenous biological rhythms to the changing daily rhythm of light and darkness and the annual fluctuation of the photoperiodicity, which

allows the organism to remain in phase with its surroundings and sustain vital biological functions such as reproduction (Falcón et al., 2010). The melatonin released into the blood affects the regulation of fish reproduction via the hypothalamus-pituitary-gonad (HPG) axis and also acts directly on the brain, pituitary, and gonads. The response to melatonin production is species-specific and can have either a pro- or antagonistic-gonadal response depending on the photoperiod, light intensity, or light spectrum cue present during the species' natural spawning season (Falcón et al., 2010).

In terrestrial vertebrates, the HPG axis mediates the secretion of gonadotropin hormones: Luteinizing hormone (LH) and Follicle-Stimulating hormone (FSH), which drive spermatogenesis. The hypothalamus, pituitary gland, and gonad operate in concert through positive and negative feedback loops while also interacting with other neuroendocrine networks, such as the hypothalamic-pituitary-thyroid axis (i.e., metabolism regulation; HPT axis) and the hypothalamic-pituitary-adrenal axis (i.e., stress response; HPA axis; Zohar et al., 2010). Consequently, gonads are highly sensitive to disruptive external factors, such as environmental, social, and nutritional stresses (Zohar et al., 2010).

Until recently, the mechanism underlying the HPG axis response to disruptive environmental factors was unclear. In mammals, the discovery of the Kisspeptin (KISS) and G-protein-coupled receptor (GPR54) signaling system has improved the understanding of how the environment interacts to regulate reproductive development, in particular for the onset of puberty (Roa et al., 2008). KISS-GPR54 signaling has been found to play a central role in regulating Gonadotropin-Releasing Hormone (GnRH) secretion from the hypothalamus (Akazome et al., 2010). GnRH subsequently triggers the secretion of LH and FSH from the pituitary gland into the bloodstream to promote gonadal development and gametogenesis (Billard, 1986). The KISS system is also involved in other key biological functions, such as nutrition, metabolism, and response to photoperiodicity. As such, the KISS system is considered a "gatekeeper" for puberty and reproduction in mammals (Tena-Sempere, 2006).

In teleosts, three KISS genes (*Kiss1*, *Kiss2*, and *Kiss3*) and four KISS receptors (*Kissr*) have been found (Tena-Sempere, 2006). While the duplication of *Kiss* genes in teleosts contrasts strongly with mammals,

where only one KISS gene is present, the role of KISS genes has been suggested to hold a similar role in teleosts in mediating the puberty process and reproductive system function (Um et al., 2010). Neurons with KISS and GnRH receptors work together as a system to modulate gonadotropin secretion in teleosts (Zohar et al., 2010). Based upon the sensitivity of the KISS system to environmental (Cowan et al., 2012; Revel et al., 2006) and behavioral (Grone et al., 2010) cues, it is now regarded as a key point of integration, linking reproductive cues to neuroendocrine circuits (e.g., HPG, HPT, and HPA) that regulate reproduction (Zohar et al., 2010).

As a further demonstration of the reproductive diversification of teleosts, up to eight GnRH variants and up to five GnRH receptors have been found (Kah et al., 2007). High GnRH duplication presents significant potential for sub-functionalization of each variant and may be responsible for driving the highly variable responses to environmental cues that are seen across different species (Lethimonier et al., 2004). Furthermore, the affinity of LH and FSH receptors is less strict in teleosts than in mammals, with receptor cross-talk observed in several species, including Atlantic salmon (*Salmo salar*), African catfish (*Clarias gariepinus)*, and Japanese eel (*Anguilla japonica*; Schulz et al., 2010). Finally, unlike in mammals, both LH and FSH receptors have been found in the steroidogenic Leydig cells of African catfish (Vischer et al., 2003) and Japanese eel (Kazeto et al., 2008), adding to the complexity of hormonal regulation pathways present in teleosts.

Ultimately, the endocrine regulation system in teleosts shows significant diversification (Billard, 1986; Knapp and Carlisle, 2011; Zohar et al., 2010; Zohar and Mylonas, 2001). While the process of spermatogenesis as a whole remains consistent, novel spermatogenic strategies have been developed exclusively in teleosts (Parenti and Grier, 2004; Schulz et al., 2010; Uribe et al., 2014). The increased plasticity of molecular mechanisms that underlie spermatogenesis in teleosts has allowed the development of highly diverse reproductive strategies in response to a variety of environmental conditions (Knapp and Carlisle, 2011; Migaud et al., 2010; Uribe et al., 2014). However, the lack of knowledge and understanding about the relationship between environmental factors and specific

reproductive strategies in most fish species of commercial interest hinders the reproduction of many species in captivity (Bromage et al., 2001; Mylonas et al., 2017, 2011; Zohar and Mylonas, 2001).

2.3 Factors influencing spermatogenesis and sperm quality

2.3.1 Light

In aquaculture, broodstock can be maintained in reproductive condition all year round by manipulating the photoperiod mimicking breeding season cues (see review Mylonas et al., 2010). Specifically, manipulating the photoperiod affects melatonin production and alters *Kiss1* and *Gnrh2* gene expression, which drives the onset of spermatogenesis. For instance, in male European sea bass (*Dicentrarchus labrax*), exposure to continuous light triggered a loss of seasonal cycling of *Kiss1* and *Gnrh2* and prevented testicular development. In contrast, exposure to the natural photoperiod occurring during the spawning season activated spermatogenesis by modulating the expression of *Kiss1* and *Gnrh2*, with *Kiss2, Kiss2r,* and *Gnrh1* levels of expression remaining constant (Espigares et al., 2017). The effect of melatonin on the reproductive system was also directly measured by exposing broodstock to exogenous melatonin treatments, which significantly increased testicular development (i.e., gonadosomatic index; GSI) and maturation (Amano et al., 2000; Aripin et al., 2014). For example, in killifish (*Fundulus heteroclitus*), exposure to melatonin significantly increased the proportion of rapidly motile spermatozoa, which increased hatching by 20% and embryonic survival by about 25% (Lombardo et al., 2014). Therefore, determining the melatonin requirement of the species of interest can be a means to improve sperm quality and spawning outcomes for aquaculture production.

While photoperiod is the main factor used to manipulate melatonin production in captivity to trigger the HPG hormonal cascade and maintain broodstock reproductive condition outside the natural breeding season, light intensity and spectrum (i.e., wavelength) are additional factors affecting melatonin production, including in the European sea bass (Bayarri et al., 2004, 2002; Vera et al., 2010), Nile tilapia (*Oreochromis niloticus*; Ridha and Cruz, 2000), Atlantic salmon (Vera et al., 2010), Atlantic cod (*Gadus morhua*; Vera et al., 2010), and yellowtail clownfish (*Amphiprion clarkii*; Shin et al., 2011).

Furthermore, Kim et al. (2016) demonstrated in olive flounder (*Paralichthys olivaceus*) that green light irradiation played a role in preventing DNA damage caused by thermal stress. Moreover, research studying the effect of light intensity and spectrum demonstrated that optimized intensity and spectrum had a positive effect on larval rearing, growth development (Blanco-Vives et al., 2010; Villamizar et al., 2009; Yamanome et al., 2009), and female reproductive condition (Bapary et al., 2011; Chattoraj et al., 2005; Shin et al., 2013; Takeuchi et al., 2011). However, the effect of light intensity and spectrum on male reproductive conditions is still unknown. Since melatonin levels are an indicator of reproductive success and, with the light spectrum having the potential to mitigate the negative effect of environmental stressors, the investigation of the effects of light intensity and spectrum on spermatogenesis and sperm quality could be an additional avenue to enhance male reproductive condition in captivity.

2.3.2 Temperature

Temperature is a critical environmental cue regulating gonadal development, sex differentiation, maturation, and spawning for many teleosts (Domingos et al., 2012). For tropical species, seasonal changes of temperature are the most important environmental cue prompting gonadal development in mature fish (Migaud et al., 2013). For instance, the elevation of water temperature has been shown to increase spermatogonial proliferation in the peppered loach (*Lepidocephalus guntea*; Majhi and Choudhary, 2014), spermatogenesis in Nile tilapia (Vilela et al., 2004), as well as sex steroid levels and GSI in pacu (*Prochilodus argenteus*; Domingos et al., 2012). In contrast, exposure to lower temperatures in pacu suppressed testicular development, lowered sex steroid levels, decreased GSI, and the number of spermatocytes and spermatids (Domingos et al., 2012). In contrast, periods of low temperature produced increased recruitment of early spermatogonia and germ cells in Nile tilapia (Melo et al., 2016). In other species, high water temperature disrupts gametogenesis, gamete maturation, and spawning, such as in meager (*Argyrosomus regius*), gilthead seabream (*Sparus aurata*), striped bass (*Morone saxatilis*), and greater amberjack (*Seriola dumerili*), and acts as a signal for ending the breeding season (Mylonas et al., 2016).

In most species, the optimal temperature for spermatogenesis and spermiation coincided with the highest hormone production levels. For instance, when some fish were injected with growth hormone, the steroidogenic activity and sex steroid production only increased when reared within an appropriate temperature range (Gopal et al., 2014; Singh and Lal, 2008). Specifically, in European eel (*Anguilla anguilla*), it was found that it was not the synthesis of androgens (i.e., testosterone and 11-keto-testosterone; 11-KT) that was affected by the water temperature, but rather oestrogen and progestin synthesis, resulting in arrested spermatogenesis (Peñaranda et al., 2016). Therefore, the process of spermatogenesis could be temperature-dependent because of the involvement of enzymes, such as cytochrome P450 aromatase, in the synthesis of oestrogen, which only takes place at a specific temperature. Moreover, it is known that temperature heavily influences the presence and state of hormones (Arfuso et al., 2017; Athauda and Anderson, 2005). While hormones are usually maintained as free steroids under normal species-specific optimal temperatures, hormones can be transformed into steroid glucuronides and rendered biologically inactive outside the optimal thermal range (Van Der Kraak and Pankhurst, 1997). It has been suggested that the temperature specificity required for reproduction and optimal larval development are the result of evolution (Manning and Kime, 1985).

While it is of commercial importance to manipulate environmental factors, such as temperature, to expand the breeding season throughout the year, the effects of manipulating temperature over a long period and/or within a suboptimal range on spermatogenesis and sperm quality are unknown. Similarly, the consequences of rearing fish under suboptimal temperatures regarding sperm quality (e.g., inappropriate long-term temperature and extreme short-term events), such as in a pond system where thermal parameters are hard to control, have not been investigated. However, advanced sperm function assessments have already shown that heat stress increases oxidative stress, reducing sperm quality and offspring viability in humans, mice, and livestock (Paul et al., 2008; Penã et al., 2017; Perez-Crespo et al., 2008). Moreover, temperature affects sperm motility and velocity, potentially resulting in fertilization issues in aquaculture (Dadras et al., 2017; Sadiqul Islam and Akhter, 2012). Therefore, understanding the effect of temperature specifically on sperm quality is necessary for aquaculture species.

2.3.3 Salinity

For marine and estuarine species, fluctuations in salinity can influence the male reproductive system. Many commercially important species, such as striped bass, European sea bass, Nile tilapia, barramundi (*Lates calcarifer*), white perch (*Morone americana*), gilthead seabream, turbot (*Scophthalamus maximus*), and Atlantic cod, are euryhaline and can tolerate a wide range of salinity. Despite this, these species still have a defined salinity range to achieve successful reproductive development and spawn.

Like photoperiod regime and temperature, salinity modulates the neuroendocrine pathways by influencing the interaction between melatonin production and the KISS-HPG axis (Falcón et al., 2010). A study of Atlantic salmon provided evidence for differential activation of the HPG axis at different salinities, where spermatogenesis was promoted by FSH exclusively in saltwater conditions and the completion of maturation was triggered by LH through decreased photoperiod (Melo et al., 2014). Additionally, an investigation on the effect of salinity on melatonin levels in European sea bass and gilthead seabream revealed that fish reared in water with a high salinity had the lowest melatonin levels, whereas the highest melatonin levels were recorded when reared in water with low salinity (Kleszczyńska et al., 2006; López-Olmeda et al., 2009). In contrast, melatonin levels in coho salmon were upregulated in salt water (Gern et al., 1984), indicating a dimorphic response of melatonin and HPG-axis to salinity across species based upon natural migration patterns between freshwater and saltwater environments.

While spawning events can be achieved across a broad range of salinities for many species, an inhibitory effect on reproductive performance was observed in suboptimal salinities. For example, in the salinity-tolerant Florida-red hybrid strain of tilapia (*Oreochromis urolepis hornorum* x *0. mossambicus*), reproductive performance was observed at high salinity (i.e., 27–36 ppt), albeit with impaired fertilization, hatching, and larval survival rates when compared to normal conditions of 1 to 18 ppt (Watanabe et al., 1989). In black bream (*Acanthopagrus butcheri*), salinity did not affect gonadal maturation; however, plasma steroid levels, sperm motility, and fertilization rates were decreased in males held in water with low levels of salinity (i.e., 5 ppt) when compared to saltwater salinity (i.e., 35

ppt; Haddy and Pankhurst, 2000). Similarly, gobiid fish (*Gillichthys mirabilis*) reared at 75 ppt for 20 days did not initiate gonadal recrudescence and had a reduced gametogenesis rate compared to when reared at 35 ppt (De Vlaming, 1971).

Although gametogenesis and spawning events often occur in suboptimal salinities for many species, the reduced reproductive performance observed might be caused by suboptimal sperm activation. In teleosts, mature spermatozoa produced within the testes are stored immotile in seminal plasma. The average osmolality of teleost' seminal plasma ranges from 200 to 400 mOsm/kg, except for sturgeons (Acipenseridae; i.e., 50–100 mOsm/kg; Alavi et al., 2011b; Alavi and Cosson, 2006). Motility activation occurs once spermatozoa are released into an external hypotonic or hypertonic aqueous environment (Morisawa, 1985). For most marine teleosts, it is the high osmolality of seawater (i.e., < 900 mOsm/kg) that creates a hyperosmotic shock, triggering sperm motility activation. In contrast, for most freshwater species, it is the low osmolality of the fresh water (i.e., > 200 mOsm/kg) that creates a hypo-osmotic shock and initiates sperm motility (Billard and Cosson, 1990). While a hyper or hypo-osmotic shock can trigger sperm motility, sperm swimming ability varies mainly in response to the osmolality of the aqueous environment. Optimal sperm activation, measured by the proportion, duration, and speed of motile spermatozoa, is achieved at a defined osmolality, which is species-specific (Alavi and Cosson, 2005). For example, in European sea bass, an osmolality of 870 mOsm/kg was the optimal activation value within the 580 to 1320 mOsm/kg range tested (Abascal et al., 2007). In pufferfish (*Fugu niphobles*), lower osmolality was linked to longer sperm motility time (Morisawa and Suzuki, 1980). However, despite the increase in motility duration, low osmolality also reduced the proportion of total and progressive motility, rendering the sperm sample improper for artificial fertilization (Gallego et al., 2013b).

In freshwater species such as in the freshwater straked prochilod (*Prochilodus lineatus*), higher sperm motility was achieved with an activation solution with an osmolality ranging from 100 to 150 mOsm/kg using glucose but not when using NaCl. However, post-thaw, both activation solutions had a similar effect on sperm motility (Viveiros et al., 2016). Similar results were seen in other freshwater species,

such as carp (*Cyprinus carpio;* Billard et al., 1995) and sterlet (*Acipenser ruthenus*; Alavi et al., 2011b). In Northern pike (*Esox lucius*), sperm motility was observed with an activation solution with an osmolality ranging from 125 to 235 mOsm/kg but was inhibited outside this range (Alavi et al., 2009). At a higher osmolality, the ionic channels of the flagellum could not operate, whereas, at a low osmolality, the integrity of the flagellum was disrupted (Alavi et al., 2009). Furthermore, some freshwater species have evolved to be specific to different environmental triggers. For example, in salmonids and some sturgeons, potassium ion (K^+) is the main factor inhibiting the activation of the spermatozoa, counteracted by ions, such as sodium (Na^+) and calcium (Ca^{2+}), whereas CO_2 is the primary inhibitor of sperm motility in turbot (Alavi and Cosson, 2006).

Collectively, these studies suggest that salinity, in contrast to light and temperature, is not a potent modulator of gonadal maturation or recrudescence in male fish. However, species-specific optimal water salinity increases reproductive performance by enhancing endocrine stimulation and sperm quality. While fish can be reared outside of their natural spawning salinity range for growth, matching conditions with those found in each species' natural habitat is necessary to achieve the most successful breeding and fertilization outcome.

2.3.4 Nutrition

Knowledge on the nutritional requirements of broodstock is limited, with studies usually focusing on the effect of nutrition on female broodstock and larval development (see review Izquierdo et al., 2001). However, several studies have demonstrated the importance of nutrition for spermatogenesis and sperm quality. Broodstock provided with nutritionally deficient diets often exhibit incomplete operation of the HPG axis, particularly when biochemical components required for gamete formation are limited (Izquierdo et al., 2001). Key nutritional elements that have been identified as influencing reproductive performance include dietary lipids, dietary protein, vitamins, and carotenoids.

Provision of insufficient dietary fatty acids decreased plasma cholesterol levels in male European sea bass, preventing significant seasonal changes in the synthesis of sex steroids, such as 11-KT, that occur

under natural conditions (Cerdà et al., 1997). European sea bass fed marine oil diets (n-3 highly unsaturated fatty acids, n-3 HUFAs) had higher expressions, in both brain and testis tissues, of 11-KT and estradiol (E_2) producing enzymes (i.e., *Cyp11b* and *Cyp19a*), and the HPG regulator *Kiss2* (Bogevik et al., 2014). Additionally, polyunsaturated fatty acids (PUFAs) in muscle, liver, and testis-supporting tissue synthesis support final gonadal maturation and energy demand during the spawning season (Baeza et al., 2014; Izquierdo et al., 2001; Martín et al., 2009). For example, male European sea bass broodstock fed the PUFA-enriched diets exhibited a longer spermiation period with higher milt and sperm density than those fed the control diets (Asturiano et al., 2001). These enriched diets did not improve sperm motility or fertilization rate; however, they did support higher embryonic and larval survival rates (Asturiano et al., 2001). In the European eel, correlations between levels of specific fatty acids in tissues and some sperm quality parameters were found. Eicosapentaenoic acid (20:5-n3) levels in the liver seemed to influence sperm volume. In contrast, the levels of α-linolenic acid (18:3-n3) and linoleic acid (18:2-n6) in the liver decreased when sperm motility increased (Baeza et al., 2015). Additionally, arachidonic acid (20:4n−6) levels decreased in muscle, liver, and testis, when sperm velocity increased (Baeza et al., 2015). These findings reinforce the key role of the liver and other tissues regarding fatty acid storage in supporting spermiation by providing an energy source to spermatozoa. In Senegalese sole, a PUFA-enriched diet effectively increased sperm quality when combined with antioxidants (i.e., vitamin E and selenium), which resulted in significantly higher fertilization rates (Beirão et al., 2015). However, Senegalese sole broodstock fed a PUFA-enriched diet without antioxidants showed no significant improvement in sperm quality and fertilization rate because the PUFA-enriched diet significantly increased lipid peroxidation in spermatozoa (Beirão et al., 2015). Additionally, despite an increase in cholesterol and unsaturated fatty acid content in spermatozoa in broodstock fed a PUFA- and antioxidants-enriched diet, sperm membrane integrity was not enhanced (Beirão et al., 2015), although the addition of cholesterol was shown to improve plasma membrane integrity in rainbow trout (*Oncorhynchus mykiss*; Müller et al., 2008). Another study of the European sea bass demonstrated that sperm motility was negatively correlated with the cholesterol/phospholipids (CHO/PL) ratio and that the CHO/PL ratio was positively correlated with lipid peroxidation (Martínez-páramo et al., 2012). Similarly, rainbow trout fed with the n-3 PUFA-enriched diet supplemented at a 2% level resulted in

the highest n-3 PUFA levels in semen and liver (Köprücü et al., 2015). However, stimulation of antioxidant defenses, resulting in lower oxidative stress, was attributed to an increase of the antioxidant capacity of the n-3 PUFA dietary supplement allowing tissues, such as the liver, to detoxify free radical damage (Köprücü et al., 2015). These results collectively demonstrate the importance of PUFAs as a key component of dietary oils that facilitate changes in sex steroid hormones, thereby affecting steroidogenesis and spermatogenesis quality (Peng et al., 2015).

Dietary lipid content has been shown to improve reproductive performance in several teleosts such as European sea bass (Fernández-Palacios et al., 1997), Atlantic halibut (*Hippoglossus hippoglossus*; Fernández-Palacios et al., 1997), gilthead sea bream (*Sparus aurata*; Fernández-Palacios et al., 1997), yellowfin sea bream (*Acanthopagrus latus*; Zakeri et al., 2009), and Nile tilapia (Fernández-Palacios et al., 1997). However, the effect of dietary lipid levels in broodstock diets has only been investigated recently regarding male reproductive system function. In male dentex (*Dentex dentex*), significant recruitment of lipids to the testis of maturing fish was observed and was correlated with increased testosterone levels and GSI (Assem et al., 2016).

Unlike dietary fatty acids and lipids, dietary proteins have been suggested not to affect catfish spermatogenesis (*Rhamdia quelen*; Reidel et al., 2010). In contrast, in female catfish (Reidel et al., 2010), rainbow trout (Watanabe et al., 1984), and sea bass (Cerdá et al., 1994), a low protein diet reduced reproductive performance (Kah et al., 1994), prevented oocyte maturation and ovulation (Navas et al., 1997). However, further studies are warranted to determine the effects of dietary proteins on the male reproductive system in other teleosts.

Dietary antioxidant requirements are high throughout reproductive development and spermatogenesis, as during the complex process of spermatogenesis, reactive oxygen species are produced as a by-product of steroidogenesis (Guerriero et al., 2014). Providing an adequate amount of dietary antioxidants is necessary to prevent damage to spermatozoa by reactive oxygen species. However, the dietary requirements of antioxidants are often overlooked when formulating broodstock diets (Izquierdo et al.,

2001). In rainbow trout, the antioxidant requirements for broodstock are estimated to be 8-fold higher than for juveniles (Blom and Dabrowski, 1995). Antioxidant compounds, such as vitamins E, C, and carotenoids (i.e., astaxanthin and β-carotene) are known scavengers of oxygen radicals. A study on the effects of dietary vitamin C in male Japanese eel broodstock demonstrated that vitamin C concentration in liver, kidney, muscle, and testes was increased significantly with incremental dietary vitamin C levels (Shahkar et al., 2015). Also, increasing dietary vitamin C levels from 32 mg/kg to 911 mg/kg of feed improved the proliferation of spermatogonia and resulted in a higher GSI (Shahkar et al., 2015). Reproductive performance of gilthead seabream, measured by egg viability, hatching, and fertilization rates, was significantly enhanced by increasing dietary carotenoid levels derived from paprika oleoresin (Scabini et al., 2011). Significant improvement in fertilization rates was suggested to result from the prevention of lipid peroxidation during spermatogenesis (Izquierdo et al., 2001; Scabini et al., 2011). In goldfish (*Carassius auratus*), supplementing diets with astaxanthin and β-carotene significantly increased seminal plasma osmolality, duration of sperm motility, and sperm concentration (Tizkar et al., 2015). While supplementation of both astaxanthin and β-carotene at 150 mg/kg resulted in significant enhancement of sperm quality, only the astaxanthin-enriched diet produced a significantly higher fertilization rate (Tizkar et al., 2015). In guppies (*Poecilia reticulata*) and convict cichlids (*Amatitlania nigrofasciata*), supplementing diets with carotenoids was revealed to have no main effect on sperm quality traits (Rahman et al., 2015; Sullivan et al., 2014). However, colorful male guppy, which had high levels of carotenoid pigmentation, produced more competitive ejaculates (i.e., spermatozoa with higher swimming speed and motility duration; Locatello et al., 2006) similarly to male redside dace (*Clinostomus elongatus*; Beausoleil et al., 2012) and Arctic charr (*Salvelinus alpinus*; Janhunen et al., 2009). The discrepancy in the effect of carotenoids between fish fed a carotenoid diet and fish natural carotenoids levels/redness coloration on sperm quality can be varied. Carotenoid pigments serve a variety of physiological functions, including immunological, antioxidant, and coloration (Clotfelter et al., 2007). Individuals displaying bright red colors have sufficient carotenoids for meeting all these physiological requirements (Clotfelter et al., 2007). On the contrary, scarcity in carotenoid pigments will lead to a trade-off between allocation to functions, favoriting immunological functions to sexual and ornamental performances (Clotfelter et al., 2007). In both Rahman et al. (2015) and Sullivan et al.

(2014), the possibility that immune challenges impaired carotenoid deposition or metabolism has not been tested and might be why carotenoid supplementation did not affect the sperm quality of guppy and convict cichlids.

The addition of antioxidants to cryopreservation extenders enhances spermatozoa resistance against oxidative stress. However, the response to the antioxidant treatment is species-specific (Cabrita et al., 2011). The use of the antioxidant amino acids, such as taurine and hypotaurine, led to a significant reduction in DNA fragmentation in gilthead seabream (Cabrita et al., 2011). In contrast, amino acid supplementation did not affect DNA fragmentation in European sea bass (Cabrita et al., 2011). The supplementation of cryopreservation extender with vitamins E and C resulted in greater sperm DNA fragmentation of European sea bass than controls, suggesting a pro-oxidant effect of the vitamin supplementation at the concentration used (i.e., 1 and 10 mM; Cabrita et al., 2011). The antioxidant supplementation of cryopreservation extender in red seabream (*Pagrus major*) showed similar outcomes to gilthead seabream (Liu et al., 2015). Adding 100 mM trehalose and 50 mM taurine antioxidants provided the most protective effect by improving frozen-thawed sperm quality (Liu et al., 2015). The other antioxidants tested, including vitamin C (25 mM), vitamin E (25 mM), and vitamin A (25 mM), were also protective but not as effective as trehalose and taurine (Liu et al., 2015).

Collectively, these studies highlight the specificity of the nutritional requirements of broodstock compared to those of juveniles. Supplementing formulated diets with dietary lipids and antioxidants is essential for male broodstock to maximize gamete production, fertilization success, embryonic survival, and larval quality. It will be of interest to further study gender-specific dietary requirements, as spermatogenesis and oogenesis follow different processes to generate mature gametes. Moreover, the development of species-specific formulated diets for broodstock might be an avenue to improve outcomes of spawning events and the reliability of seedstock supply in aquaculture. However, studies on the effect of nutrition on broodstock spermatogenesis and sperm quality are currently limited due to the lack of standardized and validated methods to conduct advanced sperm function assessments for non-model and new aquaculture species.

2.4 Advanced sperm function assessments

Advanced sperm function assessments are extensively used in bovine, porcine, ovine, caprine, equine, and poultry production (Foote, 2002). Efforts to develop and adapt sperm function assessments on these six livestock species have helped to optimize cryo-banking technology, artificial fertilization, broodstock selection, and selective breeding programs (Foote, 2002). Sperm quality assessments, including sperm count, milt volume, sperm motility, and morphology, are routinely used by the livestock industry to determine the biological capacity of spermatozoa to fertilize ova (Rodríguez-Martínez, 2007). If downstream fertility issues are observed, more advanced analyses, such as the measurements of sperm DNA integrity, mitochondria integrity, viability, and ROS, can be performed (Rodríguez-Martínez, 2007). These advanced sperm function assessments also allow quantification of the effect of handling and rearing techniques to provide new insight into standard broodstock management and husbandry guidelines (Beirao et al., 2019; Cabrita et al., 2010; Figueroa et al., 2017; Hossain et al., 2011; Torres et al., 2016).

The advancement and the transfer of reproductive technologies across different livestock industries were readily enabled, as (i) only six species are mainly used for farming and (ii) livestock species share a common reproductive feature: sexual reproduction by internal fertilization. The internal fertilization process means that competitive sperm selection occurs in a similar environment along the female reproductive tract (Lüpold et al., 2020; Parker, 2020; Weir and Rowlands, 1973). Unlike in livestock species, the variety of species raised in aquaculture use a diverse range of reproductive strategies: internal or external fertilization, involving one or more individuals per sex, and can be adapted to a particular environment (i.e., fresh water, salt water, and brackish water; De Silva et al., 2008; Fitzpatrick, 2020; Smith and Wootton, 2016). The diversification of reproductive strategies in teleosts has also driven the diversification of sperm morphometry and physiology (Billard, 1986).

The diversification of reproductive strategies and sperm biology, often species-specific, has impeded the transfer of reproductive technologies from human/livestock to aquaculture. While some

methodologies exist, the lack of standards and common guidelines to assess sperm function in teleosts further prevent the technology from being implemented by new aquaculture industries (Boryshpolets et al., 2013; Horváth et al., 2012; Rosenthal et al., 2010). Therefore, to effectively adapt advanced sperm function assessments and ultimately transform ARTs from current aquaculture industries to emerging ones, a review of current methodologies for key parameters of the sperm function is warranted.

2.4.1 Sperm concentration and motility

Sperm quality is usually defined as the ability of the spermatozoa to reach and fertilize eggs (Bobe and Labbe, 2010). Thus, sperm concentration and motility are the most common parameters investigated when assessing sperm quality, as they are commonly associated with fertilization performance (e.g., as in European sea bass; Fauvel et al., 1999, Atlantic salmon; Gage et al., 2004). Sperm concentration was traditionally recorded manually under a microscope using a haemocytometer, and sperm motility was recorded by measuring the trajectory of each spermatozoa frame by frame on recording tapes, or more commonly, by visual scoring of sperm motility on a scale ranging from 0 to 5. Although these methodologies resulted in the development of Theriogenology as a field of research, they were time-consuming, impractical on a commercial scale, and results were highly dependent on the investigator (Kime et al., 2001).

Nowadays, several alternatives to determine sperm concentration exist and have proven rapid and reliable. These include the spermatocrit (i.e., the ratio of packed spermatozoa to semen after centrifugation), spectrophotometry, flow cytometry, Coulter counter devices using electrolysis or fluorescence, and computer-assisted sperm analysis (CASA; Table 2.3). While these methods have pros and cons, the use of CASA has become the new standard due to its versatility and reliability. The introduction of CASA in the 1990s aimed to resolve the lack of objectivity inherent to manual assessments and increase the efficiency of the sperm assessment process (Verstegen et al., 2002). The benefits of using CASA software are that it can determine the sperm concentration of the sample while processing the sperm motility analysis, as each sperm sample is loaded in a chamber with a fixed volume. CASA identifies spermatozoa by analyzing the intensity of a set of pixels on the digital image

in combination with threshold parameters. This analysis is then performed on a series of frames generated by a high-speed camera. The higher the number of frames acquired per second, the higher the accuracy of the analysis will be. Once the set of pixels is identified as a centroid, CASA will track these centroids across a fixed set of frames. The coordinates on the x and y-axes are recorded on each frame and generate kinetic data to analyze the spermatozoon motion. Specifically, curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), straightness of the average path (STR; % of VSL/VAP), linearity of the curvilinear path (LIN; % of VSL/VCL), wobble (WOB; % of VAP/VCL), and beat-cross frequency (BCF) are parameters evaluated by CASA using commercial or open-source software (Figure 2.2). These motion parameters, singularly or collectively, allow the determination and classification of sperm motility. The classification of sperm motility occurs via a decision-tree-based approach. Reference laboratories, such as the World Health Organization (WHO), provide the threshold values for the classification of human sperm motility. However, these threshold values must be specifically optimized for teleost species of interest. In all cases, sperm motility data goes through a set of criteria with predetermined threshold values for each parameter to determine if spermatozoa are immotile or display slow, medium, or fast motility. A similar principle is used to identify sperm morphological abnormalities (i.e., abnormal head, acrosome defect, detached head, abnormal mid-pieces, bent tails, coiled tails, proximal droplets, and distal droplets). The classification of abnormal sperm morphologies in CASA is standardized for humans and livestock. However, it is yet to be optimized to suit the wide range of morphologies and ultra-structures of teleost spermatozoa. Furthermore, CASA can also measure sperm integrity if coupled with a fluorescence microscope, permitting the assessment of all key sperm quality parameters on a single digital imaging-based analysis system.

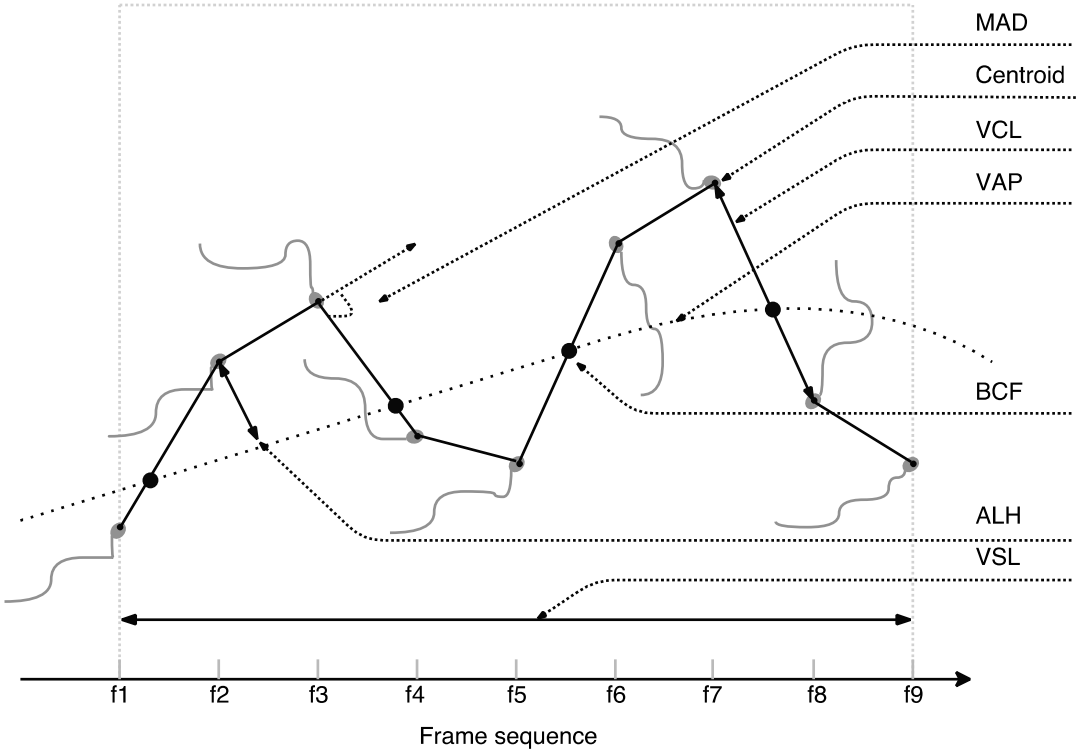

Figure 2.2: Parameters retrieved by computer-assisted sperm analysis (CASA) from video recording sperm motility. Spermatozoa are tracked over each frame and considered centroids by the CASA software. These centroids provide data on the curvilinear velocity (VCL; μm/s), straight-line velocity (VSL; μm/s), average path velocity (VAP; μm/s), amplitude of lateral head displacement (ALH; μm), beat-cross frequency (BCF; Hz), and mean angular displacement (DMA, degrees). These parameters, when combined, allow calculating the straightness of the average path (STR; % of VSL/VAP), linearity of the curvilinear path (LIN; % of VSL/VCL), and wobble (WOB; % of VAP/VCL). Figure modified from Amann and Waberski (2014).

CASA technology is commercially used for humans and livestock and is a major assessment tool in artificial insemination centers. Artificial insemination centers use CASA technology to determine sperm concentration and motility, reject non-usable ejaculates and prepare insemination doses. While advanced sperm function assessments using CASA have gained in popularity in fishery and aquaculture research, the implementation of this technology has been mainly used to monitor environmental factors, such as the effects of pollutants and water quality, and improve the reliability of cryopreservation protocols (Table 2.3). In some high commercial value and/or well-established aquaculture industries, such as cyprinid, salmonids, halibut, sea bass, seabream, and eels, CASA technology has been used to characterize the seasonal and the intra-male variability of sperm quality (Table 2.3).

Although CASA technology was developed to provide a reliable quantitative approach to assess sperm concentration and kinetics, theriogenologists raised concerns about the lack of standardization of procedures for livestock CASA assessment (Ehlers et al., 2011). In addition, inter-observer variations and variations in CASA software algorithms generate additional factors compromising the accuracy and the repeatability of the results (Ehlers et al., 2011). These variations have made the comparison of research outcomes difficult to interpret. In order to establish uniformity in the assessment of human sperm globally, the first manual on semen analysis was introduced by the WHO in 1980 (Prathima et al., 2015).

Table 2.3 Summary of techniques used to estimate sperm concentration in mammals and fish and reason for application.

Group/Species	Technique	Application	References
Mammals			
Humans and livestock	Haemocytometer Spectrophotometry Flow cytometer CASA		Brito et al. (2016)
Fish model species			
Zebrafish (*Danio rerio*)	Haemocytometer Spectrophotometry CASA	Environmental toxicity Cryopreservation	Chen et al. (2017) Yang and Tiersch (2009)
Fighting Fish (*Betta splendens*)	CASA	Environmental toxicity	Montgomery et al. (2014)
Aquaculture species			
African catfish (*Clarias gariepinus*)	Haemocytometer	Cryopreservation	Rurangwa et al. (2001)
Atlantic salmon (*Salmo salar*)	Haemocytometer Micro-haematocrit	Conservation	Piironen (1985)
Brook trout (*Salvelinus fontinalis*)	Spectrophotometry Cell counter	Semen handling technique Cryopreservation	Judycka et al. (2016) Nynca and Ciereszko (2009)
Brown trout (*Salmo trutta*)	Spectrophotometry	Cryopreservation	Judycka et al. (2016)
Coho salmon (*Oncorhynchus kisutch*)	Micro-haematocrit	Semen handling technique	Bouck and Jacobson (1976)
Common carp (*Cyprinus carpio*)	Haemocytometer	Broodstock management	Christ et al. (1996)
European eel (*Anguilla Anguilla*)	Haemocytometer Micro-haematocrit Flow cytometry CASA	Broodstock management	Sorensen et al. (2013)
Haddock (*Melanogrammus aeglefinus*)	Haemocytometer Micro-haematocrit	Broodstock management	Rideout et al. (2004b)
Ide (*Leuciscus idus*)	Spectrophotometry	Paternal contribution	
Japanese eel (*Anguilla japonica*)	Spectrophotometry	Broodstock management	Ohta et al. (2017)
Northern pike (*Esox Lucius*)	Spectrophotometry	Paternal contribution	
Rainbow Trout (*Oncorhynchus mykiss)*	Haemocytometer Micro-haematocrit Spectrophotometry	Broodstock management Semen handling technique Cryopreservation	Bouck and Jacobson (1976) Büyükhatipoglu and Holtz (1984) Ciereszko and Dabrowski (1993) Judycka et al. (2016)
Snow trout (*Schizothorax richardsonii*)	Haemocytometer Micro-haematocrit	Semen handling technique	Agarwal and Raghuvanshi (2009)
Tambaqui (*Colossoma macropomum*)	Haemocytometer	Broodstock management	Pires et al. (2017)
Whitefish (*Coregonus clupeaformis*)	Haemocytometer spectrophotometry	Semen handling technique	Ciereszko and Dabrowski (1993)
Whitefish (*Coregonus lavaretus*)	Spectrophotometry	Cryopreservation	Judycka et al. (2016)
Yellow perch (*Perca flavescens*)	Haemocytometer Spectrophotometry	Semen handling technique	Ciereszko and Dabrowski (1993)

Similar incentives were raised at the "International Workshop on the Biology of Fish Gametes" in 2010 and 2012 to consolidate guidelines and protocols to standardize common procedures for gamete quality and preservation in fish (Horváth et al., 2012; Rosenthal et al., 2010). However, with considerable variation in CASA system and experimental design used (i.e., microscope magnification, camera recording speed, and the number of replicates and fields recorded) and a lack of clarity in the reporting of the sperm motility assessment methodology in published studies, the progress of fish gamete research and the transfer of knowledge and technology to new aquaculture species remain hindered (Table 2.4).

Collectively, there is a need for species-specific studies on the standardization of CASA software, such as those performed for the European eel and zebrafish (*Danio rerio*), as the use of suboptimal parameters and different CASA systems across multiple species highly influence sperm motility outcomes (Table 2.4; Boryshpolets et al., 2013; Gallego et al., 2013b; Sorensen et al., 2013). There is also a requirement to standardize protocols within and between species to reduce background variabilities and permit the comparison with results of related studies, which ultimately will facilitate the knowledge and technology transfer to new species of interest (Gallego et al., 2013a; Horváth et al., 2012; Rosenthal et al., 2010; Wilson-Leedy and Ingermann, 2007).

Table 2.4 Microscopic metrics and parameters used to assess sperm motility in fish using computer-assisted sperm analysis (CASA).

Species	Microscope and camera		Experimental design			CASA software			References
	Magnification	Frame rate (fps)	Replicate number	Field number	Sperm number	Tracking unit per field	Variable assessed	Sperm motility classification	
Atlantic bluefin tuna (*Thunnus thynnus*)	20x	25	*n* = 3	-	-	12 s	TM, VAP	-	Suquet et al. (2010)
Atlantic salmon (*Salmo salar*)	25x	50	*n* = 3	-	-	0.5 s	TM, VCL, VAP, VSL, LIN, STR, ALH, BCF	PM: STR > 80%	Dziewulska et al. (2011)
Atlantic salmon (*S. salar*)	200x	25	*n* = 3	-	-	60 s	TM, VCL, VAP, VSL, WOB, BCF	-	Figueroa et al. (2016b)
Brook trout (*Salvelinus fontinalis*)	10x	50	*n* = 2	-	-	12 s	TM, VSL, VCL, VAP, LIN, ALH	-	Judycka et al. (2016)
Brown trout (*Salmo trutta*)	10x	50	*n* = 2	-	-	12 s	TM, VSL, VCL, VAP, LIN, ALH	-	Judycka et al. (2016) Nynca et al. (2014)
Common carp (*Cyprinus carpio*)	-	47	*n* = 1	-	-	200 frames	TM, PM, VCL, VSL, LIN, WOB, ALH, BCF.	-	Kowalski et al. (2014)
Common carp (*C. carpio*)	50x	200	-	*n* = 3 - 5	-	25 frames	TM, VCL & VSL	-	Christ et al. (1996)
European eel (*Anguilla anguilla*)	10x	60	*n* = 3	-	-	15 s	TM, PM, FA, VAP, VCL, VSL, STR WOB, ALH, BCF	-	Vílchez et al. (2016)
European perch (*Perca fluviatile*)	-	50	*n* = 3	*n* = 1	*n* = 20	5 frames	TM, VCL	-	Hatef et al. (2010)
European sea bass (*Dicentrarchus labrax*)	-	25	-	-	-	5 s	VCL, VSL, VAP, ALH, BCF	FA: VSL > 45 μm/s ME: VSL = 10 - 45 μm/s SL: VSL < 10 μm/s I: VSL = 0 μm/s	Felip et al. (2006)
European sea bass (*D. labrax*)	20x	-	-	-	-	1 s	TM, VAP	-	Fauvel et al. (2012)

Table 2.4 (continued)

Species	Microscope and camera		Experimental design			CASA software			References
	Magnification	Frame rate (fps)	Replicate number	Field number	Sperm number	Tracking unit per field	Variable assessed	Sperm motility classification	
European sea bass (*D. labrax*)	25x	25	-	-	-	12 s	VCL, VSL, VAP, TM, LIN, DMA, STR, ALH, BCF	-	Abascal et al. (2007) Kime et al. (1996)
European weatherfish (*Misgurnus fossilis*)	-	-	*n* = 2	-	*n* = 50	5 frames	TM, VCP	-	Alavi et al. (2013)
Japanese anchovy (*Engraulis japonicus*)	-	100	-	-	-	-	TM, VCP	-	Pandey et al. (2017)
Pufferfish (*Takifugu niphobles*)	10x	50	*n* = 3	-	-	-	TM, PM, VCL, VSL, VAP, STR, LIN, WOB, ALH	FA: VAP > 100 μm/s ME: VAP = 50 - 100 μm/s SL: VAP = 10 - 50 μm/s I: VCL < 10 μm/s	Gallego et al. (2013b)
Rainbow trout (*Oncorhynchus mykiss*)	10x	50	*n* = 2	-	*n* = 15	12 s	TM, VSL, VCL, VAP, LIN, ALH	-	Judycka et al. (2016)
Rainbow trout (*O. mykiss*)	10x	50	*n* = 1	*n* = 1	*n* = 15	12 s	TM, VSL, VCL, VAP, LIN, ALH	-	Dietrich et al. (2014)
Senegalese sole (*Solea senegalensis*)	10x	25	*n* = 3	-	-	-	TM, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF	I: VCL < 10 μm/s	Beirão et al. (2009)
Spotted wolffish (*Anarhichas minor*)	20x	25	-	*n* = 2	-	15 s	VCL, VSL, VAP, BCF, ALH, TM	-	Kime and Tveiten (2002)
Streaked prochilod (*Prochilodus lineatus*)	400x	25	-	-	*n* = 500	25 frames	VCL, VSL, VAP	I: VSL < 10 μm/s	Viveiros et al. (2010)
Turbot (*Psetta maxima*)	750x	-	-	-	-	-	TM, VSL, BCF	-	Dreanno et al. (1999b)
Whitefish (*Coregonus lavaretus*)	10x	50	*n* = 2	-	-	12 s	TM, VSL, VCL, VAP, LIN, ALH	-	Judycka et al. (2016)
Zebrafish (*Danio rerio*)	-	-	-	-	-	-	TM, PM, VAP, VSL, VCL	-	Chen et al. (2017)

Abbreviations: Frame per second (fps); Total Motility (TM; %); Progressive Motility (PM; %); Curvilinear Velocity (VCL; μm/s), Straight Line Velocity (VSL; μm/s), Average Path Velocity (VAP; μm/s), Straightness of the average path (STR, %), Linearity of the curvilinear path (LIN, %), wobble (WOB,%), amplitude of lateral head displacement (ALH; μm), Beat-Cross Frequency (BCF; Hz), Mean angular displacement (DMA, degrees), Fast motility (FA), Medium motility (ME), Slow motility (SL), Immotile (I).

2.4.2 Sperm viability and genetic integrity

Viability assessment is an analysis performed to determine if the spermatozoa are alive or dead via the assessment of the integrity of the plasma membrane or via the assessment of mitochondrial function. This analysis is often required to diagnose the cause of low motility in sperm samples (e.g., deficient mitochondria and plasma membrane). Viability assessment is usually performed using a dual staining method, where a membrane-permeant dye (i.e., SYBR 14 or Hoechst 33258) stains DNA in living cells and a membrane-impermeable dye (i.e., propidium iodide; PI) stains non-viable spermatozoa: the intact membrane in viable spermatozoa will prevent PI to bind with DNA (Table 2.5). A similar dual staining approach is used to assess mitochondrial function, where a cell-permeant dye such as Rhodamine 123 (RH123) stains active mitochondria, and a membrane-impermeant dye such as PI is used to evaluate the membrane integrity of the spermatozoa (Table 2.5). This mitochondrial assessment aims to determine if the spermatozoa's immobility (or the general low motility rate) originated from a lack of energy supplied by the mitochondria or a lack of plasma membrane integrity.

New stains have been recently used in teleosts to investigate spermatozoa at different stages of membrane integrity. The dual stain Annexin V-FITC/PI, where Annexin-V is a calcium-dependent probe, is used to track the externalization of phosphatidylserine (PS) located at the inner face of the lipid bilayer for intact spermatozoa. This dual staining method has been tested on spermatozoa of Senegalese sole to detect membrane instability (Table 2.5; Beirão et al., 2009, 2008). A similar approach using the dual stain YO-PRO-1/PI has also been tested on spermatozoa of Senegalese sole, where YO-PRO-1 is used as a DNA marker for cells with a compromised plasma membrane (Table 2.5; Valcarce et al., 2016). YO-PRO-1 stains apoptotic spermatozoa that remain impermeable for PI, and viable spermatozoa remain unstained. YO-PRO-1, thus, distinguishes between dying spermatozoa and dead spermatozoa. More advanced staining techniques have emerged using a triple stain method to resolve the issue of unstained sperm subpopulations present with the dual stain approach (i.e., YO-PRO-1/P1 and Annexin V-FITC/PI). The triple combination of SNARF-1/YO-PRO-1/ethidium homodimer or Merocyanine 540/YO-PRO-1/Hoechst 33258 stains allows to label all spermatozoa and provides a complete characterization of the viability of the sperm sample. The use of an additional dye revealing the

intermediate stage between either living or dead status provides crucial information, as a spermatozoon undergoing an early apoptotic event cannot fertilize an egg despite being alive and having high motility. This information is particularly important in order to quantify cryoinjury during the elaboration of an optimal cryoprotectant formula. However, this triple staining method to assess sperm viability has not been tested on spermatozoa from teleosts to date, but is established in mammals (Kavak et al., 2003; Peña et al., 2007, 2005).

Table 2.5 Viability and mitochondrial status assessments using dual staining method applied to sperm samples in teleosts.

Species	**Staining method**	**Technique**	**Sperm viability**	**References**
Atlantic salmon (*Salmo salar*)	CFDA SE/PI	Flow cytometry	-	Honeyfield and Krise (2000)
Atlantic salmon (*S. salar*)	SYBR 14/PI	Flow cytometry	Live (fresh): 90.5 ± 3.9% Live (post-thaw): 75.2 ± 6.3%	Figueroa et al. (2016b)
Brown trout (*Salmo trutta*)	PI	Spectrofluorometer	-	Labbe and Maisse (2001)
Common carp, (*Cyprinus carpio*)	SYBR 14/PI	Fluorescence microscopy	Live: 93-100%	Flajšhans et al. (2004)
European sea bass (*Dicentrarchus labrax*)	SYBR 14/PI	Fluorescence microscopy	Live: 44%	Cabrita et al. (2011)
Gilthead seabream (*Sparus aurata*)	SYBR 14/PI	Flow cytometry	Live: 77%	Cabrita et al. (2005a)
Gilthead seabream (*S. aurata*)	SYBR 14/PI	Fluorescence microscopy	Live: 58%	Cabrita et al. (2011)
Gray snapper (*Lutjanus griseus*)	SYBR 14/PI	Flow cytometry	Live: 44-75%	Riley (2002)
Lake trout (*Salvelinus namaycush*)	CFDA SE/PI	Flow cytometry	-	Honeyfield and Krise (2000)
Nile tilapia (*Oreochromis niloticus*)	SYBR 14/PI	Flow cytometry	Live: 82 ± 10%	Segovia et al. (1999)
Nile tilapia (*O. niloticus*)	RH123/PI	Flow cytometry	Live: 78 ± 14%	Segovia et al. (1999)
Rainbow Trout (*Oncorhynchus mykiss*)	RH123/PI	Flow cytometry	Live: 99%	De Baulny et al. (1997)
Rainbow Trout (*O. mykiss*)	CFDA SE/PI	Flow cytometry	-	Honeyfield and Krise (2000)
Rainbow Trout (*O. mykiss*)	SYBR 14/PI	Flow cytometry	Live: 56.0 ± 9.4% Dying: 18.1 ± 3.4% Dead: 25.9 ± 6.7%	Dietrich et al. (2014)
Rainbow Trout (*O. mykiss*)	SYBR 14/PI	Flow cytometry	Live (fresh): 98.6 ± 0.4% Live (post-thaw): 37.6 ± 1.4%	Pérez-Cerezales et al. (2010)
Rainbow Trout (*O. mykiss*)	SYBR 14/PI	Flow cytometry	Live (fresh): 99.1 ± 5.1% Live (post-thaw): 98.4 ± 1.4%	Figueroa et al. (2013)
Rainbow Trout (*O. mykiss*)	JC-1	Flow cytometry	Live (fresh): 68.4 ± 3.4% Live (post-thaw): 36.2 ± 5.4%	Figueroa et al. (2013)
Red snapper (*Lutjanus campechanus*)	SYBR 14/PI	Flow cytometry	Live: 47-82%	Riley (2002)
Senegalese sole (*Solea senegalensis*)	Annexin V-FITC/PI	Fluorescence microscopy	Dying: 6-23%	Beirão et al. (2009)
Senegalese sole (*S. senegalensis*)	PI/YO-PRO-1	Flow cytometry	Dying: 25-27%	Valcarce et al. (2016)
Senegalese sole (*S. senegalensis*)	Annexin V-FITC/PI	Fluorescence microscopy	Live: < 25% Dying: 20%	Beirão et al. (2008)
Siberian sturgeon (*Acipenser baerii*)	SYBR 14/PI	Fluorescence microscopy	Live: 80-96%	Flajšhans et al. (2004)
Tench (*Tinca tinca*)	SYBR 14/PI	Fluorescence microscopy	Live: 72-99%	Flajšhans et al. (2004)
Wels (*Silurus glanis*)	SYBR 14/PI	Fluorescence microscopy	Live: 76-85%	Flajšhans et al. (2004)

Abbreviations: carboxyfluorescein diacetate, succinimidyl ester (CFDA SE), propidium iodide (PI), cyanine dye (SYBR 14), Rhodamine 123 (RH123), cyanine dye (JC-1), Fluorescein (FITC).

Genetic integrity assessment in teleost spermatozoa gained popularity during the past decade due to an increase in knowledge of sperm genome and epigenome integrity and its relevance to the paternal genetic contribution in the development of viable embryos (Herráez et al., 2017). There are many DNA integrity assessment methods available, including Comet assay, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), sperm chromatin dispersion test (SCD), and sperm chromatin structure assay (SCSA), which provide a holistic diagnosis by evaluating the presence or absence of DNA damage in a spermatozoon. Comet assay and TUNEL are the two main approaches used to analyze sperm DNA integrity in teleosts (Table 2.6). Comet assay reveals the existence of single and double-strand breaks in individual cells and emphasizes the single-cell accuracy of the testing (Cabrita et al., 2010; Figueroa et al., 2016a). In contrast, TUNEL allows direct detection of DNA breaks and permits the processing of a large number of cells via the use of flow cytometry or fluorescent microscopy (Cabrita et al., 2010; Figueroa et al., 2016a). The benefit of these methods is that they provide a quick diagnosis of an individual's sperm DNA integrity status. Therefore, the application of these DNA integrity assessments is particularly relevant for aquaculture as; (i) broodstock in captivity are subject to numerous stressors, promoting oxidative damage in spermatozoa, and (ii) the presence of sperm DNA damage can remain undiagnosed with traditional methods as spermatozoa with DNA damage can still be highly motile and fertilize eggs (Figueroa et al., 2020; Migaud et al., 2013; Mylonas et al., 2017).

Sperm DNA damage can occur during meiosis, chromatin remodeling, and maturation in the sperm duct. Spermatozoa held in the sperm duct are particularly sensitive to DNA damage. At this stage, they no longer have DNA repair mechanisms and rely exclusively on the antioxidant properties of the seminal plasma to overcome lipid peroxidation damage. Sperm DNA damage can also occur post-collection during handling, chilled storage, and cryopreservation (Figueroa et al., 2020; Migaud et al., 2013; Mylonas et al., 2017; Shammas, 2011). More advanced methods to quantify sperm DNA damage exist, such as Comet assay/Fluorescence In Situ Hybridization (FISH), DNA Breakage Detection-Fluorescence In Situ Hybridization (DBD-FISH), and quantitative polymerase chain reaction (qPCR), and provide a quantitative measure of sperm DNA lesions for a specific sequence or genome-wide. The use of these advanced methods has permitted us to deepen our understanding of DNA injuries,

demonstrating that eggs fertilized with DNA-damaged spermatozoa lead to embryonic arrest (Fatehi, 2006), alteration in the transcription rates of genes involved in growth and development (Pérez-Cerezales et al., 2011; Cartón-García et al., 2013), abnormal growth and change in cortisol response to acute stress (Hayes et al., 2005), and haploidy (Horváth et al., 2007). The inclusion of a DNA damage assay as part of sperm quality assessment of captive broodstock has the potential to play a crucial role in developing quality bio-banking and optimizing the quality of aquaculture production.

Table 2.6 DNA damage assessment applied to spermatozoa of fish species used in aquaculture.

Species	**Technique**	**Application**	**DNA damage (%)**	**References**
Atlantic salmon (*Salmo salar*)	TUNEL	Cryopreservation	Fresh: 7.8 ± 2.1% Post-thaw: 9.2 ± 2.7%	Figueroa et al. (2015)
Common carp, (*Cyprinus carpio*)	Comet assay	Cryopreservation	Fresh: - Post-thaw: 0.0 ± 0.0%	Öğretmen et al. (2015)
European sea bass (*Dicentrarchus labrax*)	Comet assay	Cryopreservation	Fresh: 32.7 ± 11.1% Post-thaw: 38.2 ± 11.2%	Zilli et al. (2003)
Gilthead seabream (*Sparus aurata*)	Comet assay	Cryopreservation	Fresh: 28.2% Post-thaw: 31.3%	Cabrita et al. (2005b)
Gilthead seabream (*S. aurata*)	Comet assay	Cryopreservation	Fresh: - Post-thaw: 2.5 ± 0.2%	Cartón-García et al. (2013)
Rainbow Trout (*Oncorhynchus mykiss*)	Comet assay	Cryopreservation	Fresh: 11.2% Post-thaw: 30.3%	Cabrita et al. (2005b)
Rainbow Trout (*O. mykiss*)	Comet assay	Cryopreservation	Fresh: 32 ± 1.2% Post-thaw: 49.2 ± 1.1%	Pérez-Cerezales et al. (2010)
Rainbow Trout (*O. mykiss*)	TUNEL	Cryopreservation	Fresh: 8.1 ± 2.3% Post-thaw: 11.1 ± 3.9%	Figueroa et al. (2013)

Abbreviations: Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

2.4.3 Sperm proteomic characterization

Proteomics is a new tool for reproductive assessment that allows the identification and characterization of proteins as well as quantitative evaluation of their expression (Ciereszko et al., 2017). The analyses of sperm and seminal plasma proteins allow a better understanding of the mechanism involved during spermatogenesis and its interaction with extrinsic factors (Ciereszko et al., 2017). Research on the proteome is particularly pertinent in teleosts. During spermatogenesis, transcription and translation are suppressed, suggesting that molecular events involved in protein synthesis play an essential role in the spermatozoa's regulation and physiological functioning (Ciereszko, 2008).

Acute-phase proteins (APPs) are major seminal plasma proteins, including apolipoproteins and transferrin (Dietrich et al., 2014b; Nynca et al., 2014). APPs have been identified to play an essential role in protecting spermatozoa membrane integrity from oxidative damage and providing energy resources (Dietrich et al., 2011; Dietrich et al., 2014b). Additionally, APPs, such as Warm acclimation protein 65 (Wap65), have been identified as major acute immune response proteins in seminal plasma from common carp and Eurasian perch (*Perca fluviatilis*) that arise from extrinsic stresses (Dietrich et al., 2014b; Shaliutina et al., 2012). Proteomic analysis in carp spermatozoa identified 348 proteins, of which 32% were related to metabolism and energy production, 30% were associated with transcription, transport, and protein turnover, and 10% were associated with cell cycle, apoptosis, and oxidative stress (Dietrich et al., 2014a). The sperm protein assemblage was dominated by proteins involved in the metabolic process in both carp and rainbow trout (Dietrich et al., 2014a; Nynca et al., 2014). The high abundance of metabolic proteins is congruent with the high energy demand required for sperm movement, as sperm motility is mainly dependent on stored ATP and ATP synthesized by mitochondrial respiration (Perchec et al., 1995). In carp and rainbow trout, the proteomic profiles of seminal plasma and spermatozoa share 15% and 25%, respectively (Dietrich et al., 2014a). The presence of multitasking proteins (i.e., apolipoproteins, transferrin, and serum albumin) and proteins related to the immune system in both seminal plasma and sperm proteomes are likely to be the result of the adsorption of seminal plasma proteins on sperm surface to protect from proteolytic or oxidative attacks (Ciereszko et al., 2017). However, the presence of proteins, including cytoskeletal proteins and creatine

kinase, in the seminal plasma might be due to leakage from damaged or apoptotic spermatozoa (Ciereszko et al., 2017; Dietrich et al., 2014a).

The proteomic analysis of carp spermatozoa reveals differences in protein composition compared to the rainbow trout protein profile (Dietrich et al., 2014a). For instance, in carp spermatozoa, the abundance of proteins, such as NKEF-B (i.e., a protein involved in protecting spermatozoa against oxidative stress) and Wap65, is higher than in rainbow trout (Dietrich et al., 2014a). Moreover, the ability of carp spermatozoa to survive cryopreservation was associated with Wap65 (Dietrich et al., 2017). The heat shock proteins HSP90, HSP70, and HSP60, are among the ten most abundant proteins in carp spermatozoa and are prominent proteins involved in protecting stress-induced damage (Dietrich et al., 2014a). Heat shock proteins, including HSP90, HSP70, and HSP5, are also found in carp seminal plasma (Dietrich et al., 2014b). The increased presence of proteins involved in protecting spermatozoa against stress-related injuries might originate from the warm-water carp rearing condition. Hence, it would also explain their absence in sperm and seminal plasma protein profiles of rainbow trout. This variation in protein profile might be relevant to improving sperm handling techniques for short- and long-term storage and artificial fertilization.

So far, among the teleosts, only the proteomic profiles of seminal plasma and/or spermatozoa of carp, Eurasian perch, and rainbow trout have been studied. However, the species-specific characterization of the protein profile and pattern in seminal plasma and spermatozoa is a promising technique that might reveal potential pre-markers of stresses and biomarkers of sperm integrity. The selection of relevant proteins would provide a detailed evaluation for cryoinjuries and allow refinement of cryopreservation methodology. Combining these new biomarkers with other advanced sperm function assessments, including sperm DNA integrity and viability, would facilitate the improvement of sperm handling and storage techniques and broodstock management procedures (Ciereszko et al., 2017).

2.5 The significance for the aquaculture industry

A major bottleneck for many species in aquaculture is the inability to forecast spawning outcomes (i.e., number of larvae produced and their genetic diversity). It generates uncertainty regarding the capacity to meet industry demand for seedstock sustainably and creates a major financial risk for hatcheries. This issue also potentially affects the genetic gain per generation that can be achieved in selective breeding programs, preventing further industry development. While a myriad of environmental factors and endocrine disruptors can cause reproductive issues in captive broodstock, the lack of quantitative tools and methodologies available to investigate the reproductive condition of teleosts are often the main impediment preventing the gaining of a greater understanding of the species biology and physiology, as well as improving current hatchery practices.

Over the last few decades, the scientific community has focused on resolving these reproductive issues by investigating maternal reproductive capability. However, recent investigations characterizing paternal effects on offspring revealed that male reproductive condition and sperm quality are important for producing high-quality seedstock. A study on two freshwater species, Ide (*Leuciscus idus*) and Northern pike (*Esox lucius*), found that 20% of the total variance in embryonic phenotype was due to paternal effects. Thus, paternal effects were 2-fold higher than the maternal effects in Ide and were 15-fold higher than the maternal effects in Northern pike (Siddique et al., 2017). Moreover, a spermatozoon provides more than just genomic information to the egg. Paternal epigenetic information has been correlated to play a relevant role in embryonic development (Fernández-Díez et al., 2015). Alteration of either the sperm genome and/or epigenome has been associated with embryos with lower chances of survival (Aitken et al., 2014; Fernández-Díez et al., 2015; Pérez-Cerezales et al., 2010). For example, the fertilization of eggs with cryopreserved and DNA-damaged spermatozoa increased the abortion rate during gastrulation (Pérez-Cerezales et al., 2010); similar results were obtained for common carp (Öğretmen et al., 2015), tench (*Tinca tinca*; Rodina et al., 2007), and zebrafish (Gosálvez et al., 2014). The significant relationship between abnormal development of progeny and sperm DNA damage level was demonstrated to be driven by the lack of defenses of the male genome against the genotoxic

potential of environmental disturbances (Santos et al., 2013). The implication of these findings for the aquaculture industry is of utmost importance, highlighting the criticality of assessing DNA integrity in male broodstock as part of routine sperm quality assessment processes. The use of DNA integrity as selection criteria for male broodstock may help obtain better embryonic survival rates. It may also provide a better insight into the heavily skewed levels of paternity seen in mass spawning species of commercial importance, such as barramundi (Loughnan et al., 2013), common snook (*Centropomus undecimalis*; Rhody et al., 2014), Japanese flounder (*Paralichthys olivaceus*; Sekino et al., 2003), common sole (Blonk et al., 2009), Atlantic cod (Herlin et al., 2008), European sea bass (Loncar et al., 2014), Atlantic salmon (Jones and Hutchings, 2002), and gilthead seabream (Brown et al., 2005). The use of DNA integrity assessments could also provide a better insight into the low progeny survival rates resulting from using pooled milt when artificially fertilizing Atlantic halibut (Mommens et al., 2010). As such, the varied fertilization rates ranging from 1.4% to 75.6% in Chinook salmon (*Oncorhynchus tshawytscha;* Withler, 1988) and from 6.6% to 96% in northern pike (Babiak et al., 1997) resulting from the use of pooled milt may be linked to inter-male variation in sperm DNA integrity. The variability in artificial fertilization results when using pooled milt, and the skewed paternal contribution to offspring observed when breeding mass-spawning species in captivity might be linked to poor sperm DNA integrity of some individuals. Despite using large numbers of broodstock to minimize undesirable effects, such as random genetic drift and inbreeding, the poor sperm DNA integrity of some individuals might potentially cause the loss of genetic diversity commonly observed. The implementation of sperm DNA integrity assessment for broodstock could, therefore, reveal valuable information allowing the improvement of seedstock quality and increase in reliability of supply.

The characterization of broodstock reproductive capability using advanced sperm function assessments has already been implemented in several species of commercial importance. It has provided valuable information on the reproductive state of broodstock, highlighting high inter-male variability in sperm quality. For example, sperm quality evaluation in Senegalese sole revealed significant variation in sperm concentration, motility, and DNA damage between broodstock (Beirão et al., 2009; Cabrita et al., 2006). In rainbow trout, analyses of sperm motility and morphology revealed significant inter-male

variation in swimming speeds and head length and that males with short sperm heads maintained longer swimming ability than those with long heads (Tuset et al., 2008). Similar inter-male variation in sperm length, mean velocity, and ejaculate longevity was found in Atlantic salmon (Gage et al., 2002). Finally, the sperm quality assessment of post-thawed spermatozoa from Siberian sturgeon (*Acipenser baerii*) showed significant inter-male variation in ATP content (Billard et al., 2004). Despite being limited to a small number of teleost species, the use of advanced sperm function assessments has demonstrated the presence of inter-male variation in sperm quality. These results reflect similar inter-male variabilities in sperm quality observed in other taxa, such as mammals, reptiles, and insects (Helfenstein et al., 2008; Hunter and Birkhead, 2002; Khandwala et al., 2017; Rudin-Bitterli et al., 2020). Knowing intra-male variation in sperm traits for a commercial species of interest can provide a baseline for decision making that could help select broodstock for spawning events and new individuals for becoming broodstock.

While the effects of sperm quality and inter-male sperm competition on fertilization success in captivity are well demonstrated, additional post-mating mechanisms such as cryptic female choice can also skew paternal contribution during mass-spawning toward specific individuals (Gasparini et al., 2020). Specifically, females can alter sperm competition/performance by targetting specific phenotypes to suit their reproductive interest (e.g., genetic diversity, compatibility/preferred spermatozoa) using mechanisms such as female reproductive fluid to modulate sperm traits (e.g., sperm velocity; Gasparini et al., 2020). Therefore, initial characterization of male reproductive capability using advanced sperm function assessments is a prerequisite to investigate other factors that could affect fertilization success and individual reproduction performance in captive environments.

In terrestrial vertebrates, the implementation of advanced sperm function assessments to characterize male reproductive capacity has largely been implemented. The livestock industry has endorsed such technology to ensure the selection of the best broodstock for purposes, such as selective breeding programs, and ensure the sustainability of cryopreservation procedures of spermatozoa over time for gene banking purposes artificial fertilization programs. It has been shown that reproductive capability

is a heritable trait in various species, such as beef cattle (Corbet et al., 2013), stallion (Parlevliet et al., 1994), rooster (Soller et al., 1965), black tiger prawn (*Penaeus monodon*; Macbeth et al., 2007), fly (Ward, 2000), and zebra finches (*Taeniopygia guttata*; Knief et al., 2017). Moreover, a reduction in reproductive performance in domesticated lines is attested by farmers as early as the first generation for male Senegalese sole (Forne et al., 2009) and Atlantic salmon (Fleming et al., 1994). A reduction in reproductive capability is mainly present in species that have been subject to intense selective breeding, such as dairy and the Belgium blue cattle breeds, which are, nowadays, impeded by significant difficulties to procreate (Macbeth et al., 2007).

Collectively, this information highlights the relevance of implementing advanced sperm function assessments for broodstock selection in aquaculture and the importance of fostering subsequent research to identify sperm quality in teleosts. While the reproductive capacity of male broodstock of some species of commercial interest has been successfully assessed, the standardization of validation process guidelines and advanced sperm function assessments will offer a valuable tool of quality and production controls to established and emerging aquaculture industries.

2.6 Conclusion

This review highlights the complexity and variety of the reproductive system of male teleosts and its multiple interactions with the environment. The importance of the environment in regulating spermatogenesis and producing high-quality spermatozoa via the endocrine system is well established. It is species-specific, reflecting the variability of the species' natural environmental conditions. This variation in the reproductive system and sensitivity to environmental factors contrasts with terrestrial vertebrates, rendering knowledge of male fertility, advanced sperm function assessments, and advanced reproductive technology not transferable between species. Despite emerging initiatives to standardize procedures for sperm function assessments, the reliability and the accuracy of the procedures/methodologies should be validated for the species of interest and thoroughly detailed in studies to improve reproducibility and inter-species comparative analyses. This review also highlights

the recent findings on the importance of paternal contribution in post-fertilization processes, the importance of the proteome in sperm development, and the importance of the sperm genome and epigenome in larval development and survival. Future research should be emphasized towards understanding the specific role of genomic and proteomic processes on sperm quality to deepen our knowledge of spermatology in teleosts and ultimately provide new biomarkers of sperm quality. Moreover, developing a more holistic approach and standardization of advanced sperm function assessment will greatly benefit both established and emerging aquaculture industries.

Chapter 3. Validation of advanced tools to evaluate sperm function in barramundi (*Lates calcarifer*)

3.1 Abstract

Barramundi *(Lates calcarifer)* is a tropical finfish species rapidly growing in popularity for aquaculture production. However, sperm quality tests have yet to be adapted to enable the selection of highly fertile male broodstock in this species. Accordingly, in this study, advanced tools were optimized to evaluate barramundi sperm function to facilitate the future study of male fertility and address some of the reproductive constraints currently observed in captive-bred broodstock. Sperm morphology data were used to calibrate and validate automated sperm counting and motility detection by computer-assisted sperm analysis (CASA; AndroVision, Minitube). Several parameters were examined to determine the optimum settings for accurate CASA sperm counting and were compared to a manual haemocytometer method, including the sample dilution (1:1000, $r = 0.87$), the minimum number of fields ($n = 4$, CV = 7.5 %), and the effect of motile *vs.* immotile spermatozoa on automated counting (no effect, $r = 0.99$, $P < 0.001$). Assays for cell viability and DNA integrity were also validated for barramundi spermatozoa using 70 °C heat-treated controls and a 5-point intact: damaged dilution curve ($r = 0.98$, $P < 0.001$), and DNase-treated sperm controls, respectively. Data from these optimized assessments indicated high variation between individuals for each parameter assessed and the presence of high rates of DNA and membrane damage in sperm samples tested. Further research building upon this preliminary sperm quality data is required to identify the cause of DNA and membrane damage in barramundi spermatozoa and understand any potential relationships with paternal performance in commercial spawns.

3.2 Introduction

Barramundi (Asian sea bass, *Lates calcarifer*) is an emerging and promising species for aquaculture worldwide (Savage, 2016). However, improvements in broodstock and hatchery practices are required to support the continued intensification of barramundi production and support the demand for premium quality seedstock (Robinson et al., 2010).

Barramundi is a mass-spawning species involving the simultaneous release of gametes (i.e., spermatozoa and oocytes) from multiple males and females during a spawning event (Jerry, 2013). Although mass-spawning is an effective strategy to produce large quantities of offspring, the paternal contribution to subsequent larval cohorts is observed to be highly skewed in aquaculture systems (Frost et al., 2006; Loughnan et al., 2013; Wang et al., 2008). Collectively, these studies indicate that males often contribute unequally to spawns, suggesting the potential for variation in fertility between captive-bred male barramundi broodstock, the occurrence of competitive spawning behavior (Fessehaye et al., 2006), and/or sperm competition (Stoltz and Neff, 2006). The optimization of quantitative methods to assess sperm quality in barramundi is therefore critical to identify the cause of skewed contributions and also facilitate the selection of males with higher quality gametes (Loughnan et al., 2013; Macbeth and Palmer, 2011; Robinson et al., 2010).

Commonly in aquaculture, sperm quality parameters have been assessed by manual microscopy, making sperm evaluation both time-consuming and subject to high intra- and inter-observer variation (Brito et al., 2016; Palme et al., 2017). More recently, considerable effort has been invested in automating and standardizing procedures for sperm quality evaluation through the use of computer-assisted sperm analysis (CASA; i.e., sperm count and motility) and flow cytometry (i.e., viability, DNA, and mitochondrial integrity; see review Figueroa et al., 2016a). The use of molecular assays combined with CASA to assess sperm function has provided an increased understanding of teleost fertility. Live/dead cell viability assays that determine the integrity of the plasma membrane, or measure mitochondrial integrity as well as DNA integrity assays (i.e., TUNEL, SCSA/SCDA, and Comet assays)

can help identify the cause of poor fertilization or low sperm motility (Beirão et al., 2009, 2008; Cabrita et al., 2011, 2005a; Dietrich et al., 2014; Figueroa et al., 2019, 2017, 2016a, 2016b, 2013; Pérez-Cerezales et al., 2010; Valcarce et al., 2016). Collectively, with the use of sperm DNA integrity assays (i.e., TUNEL, SCSA/SCDA, and Comet assays), insight into the developmental competence of newly fertilized embryos can be investigated (Paul et al., 2008; Pérez-Cerezales et al., 2011). In teleosts, several studies have demonstrated the role of DNA damage in spermatozoa on arrested embryo development, abnormal growth, cancer formation, and morphological abnormalities in adulthood, which are recurrent issues seen in the aquaculture industry (Cartón-García et al., 2013; Fatehi, 2006; Gosálvez et al., 2014; Hayes et al., 2005; Herráez et al., 2017; Öğretmen et al., 2015; Pérez-Cerezales et al., 2011; Rodina et al., 2007; Shammas, 2011). Early detection of males with high sperm DNA damage allows screening of broodstock based upon fertility as well as the optimization of sperm handling protocols for use in assisted reproductive technologies (e.g., artificial fertilization).

Sperm characteristics and reproductive strategies in fish are complex and diverse (Billard, 1986; Mattei, 1991), limiting the horizontal transfer of sperm evaluation technologies across species. As such, the development and optimization of CASA detection parameters, staining procedures, and flow cytometry parameters are required for each species. Furthermore, the great variation in CASA detection parameters, data acquisition methods, microscope systems, and experimental designs used across different studies limits the direct comparison of results between studies (Gallego et al., 2013a; Horváth et al., 2012; Rosenthal et al., 2010; Wilson-Leedy and Ingermann, 2007) and impedes the development of species-specific procedures and experimental designs.

Therefore, this study aimed to validate advanced tools to evaluate sperm function in barramundi. More specifically, this study aimed to (i) characterize normal barramundi sperm morphology to develop a CASA detection profile, (ii) validate the use of CASA to measure sperm count and motility, and (iii) validate a sperm viability assay and DNA integrity assay using flow cytometry.

3.3 Methods

3.3.1 Broodstock

Captive-bred barramundi were maintained in a broodstock facility at James Cook University, Townsville, Australia. Male broodstock were between 2 and 4 years old, and females between 5 and 8 years old. Fish were mature adults and maintained in breeding conditions in 28,000 L tanks held in standard maturation conditions (i.e., 30 °C, 30 ppt salinity, and 16 h light: 8 h dark cycle). Fish were fed 4 days per week at 1% body weight per feed with a formulated maturation diet (LANSY-Breed M, INVE Aquaculture). As the optimization of each trial/assay required an iterative approach, milt samples were obtained on multiple occasions alternating between two broodstock cohorts composed of 8 males and 4 females each. The average weight and length of males used for milt collection were 4.4 ± 0.5 kg and 71.2 ± 0.6 cm. Experiments were approved by the James Cook University Animal Ethics Committee (A2406).

3.3.2 Sperm collection and processing

Milt was collected from males in spawning conditions. Selected broodstock were sedated in an anaesthetic bath containing iso-eugenol at 40 mg/L (AQUI-S®, New Zealand) prior to sperm collection (Schipp et al., 2007). The gonopore of males was rinsed with distilled water and dried to prevent salt water prematurely activating spermatozoa during collection. Milt samples were obtained by cannulation with a 1.72 mm diameter cannula (PE-90 polyethylene tubing, Becton Dickinson, Sparks, MD, USA), and an aliquot visually assessed by bright field microscopy for quality control (i.e., presence of blood, urine contamination, premature sperm activation, or failure to activate on contact with saltwater). Milt was aspirated into an Eppendorf tube and an aliquot diluted in an artificial extender (see below) to assess sperm count and motility within ~2 min of collection, while milt samples for viability and DNA integrity were kept raw on ice until completion of the milt collection from all males (~6 h) before being assessed.

The non-activating medium (NAM) used to dilute sperm samples was a modified Marine Ringer's solution consisting of 180 mM NaCl, 5.1 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $NaHCO_3$, 2.6 mM $NaH_2PO_4 \cdot 2H_2O$, and 5.6 mM D^+ glucose, adjusted to pH 7.4 (based on Palmer et al., 1993). The osmolality of the NAM was adjusted to 400 mOsm/kg by increasing the concentration of NaCl from 124.1 to 180 mM to match the osmolality of broodstock milt (MRS400; see Chapter 4). Sperm motility was activated using salt water consisting of 404.9 mM Na^+, 459.1 mM Cl^-, 10.1 mM K^+, 9.7 mM Ca^{2+}, 0.1 mM PO_4^{-3}, pH 8.1, and 900 mOsm/kg. All reagents were sourced from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise stated.

3.3.3 Sperm evaluation

Collected samples were evaluated for milt volume and sperm characteristics (i.e., sperm concentration, total count, morphology, motility, viability, and DNA integrity).

3.3.3.1 Volume measurement

Milt volume (*V*) was calculated by measuring the length of raw sperm in the cannula according to:

$$V = \pi * r^2 * h$$

Where *r* is the radius of the cannula (*r* = 0.43 mm) and *h* is the sample length measured in millimeters.

3.3.3.2 Morphology assessment

To assess sperm morphology, sperm samples were collected from three male broodstock. Spermatozoa were diluted 1:500 in fresh 2% paraformaldehyde (PFA) in MRS400 solution pH 7.4. A 10 µL aliquot was smeared on a glass slide and allowed to air-dry at room temperature for 30 min. Slides were subsequently immersed in SpermBlue® (Microptic, SL, Barcelona, Spain) fixative and stain solution for 2 min at room temperature before gently dipping the slide in distilled water for 5 s. Slides were permanently mounted using DPX medium (BDH Chemicals Ltd., Poole, England). Slides were prepared in triplicate for each fish and visualized by bright field illumination at 50 X final magnification (0.5 X C-Mount adaptor coupled with a 100 X objective) with an Olympus BX53 microscope

(Olympus, Tokyo, Japan). Micrographs of barramundi sperm were captured with a Basler avA1000-100gc camera (Basler AG, Ahrensburg, Germany), and morphological features were measured using ImageJ analysis software (Version 1.41; http://rsbweb.nih.gov/ij/). ImageJ was initially calibrated to translate pixels into µm scale using a micrometer glass slide. Morphological features including total sperm length, tail length, head length (L), and head width (W) were measured using ImageJ's freehand tool, while other parameters were calculated as the head ratio (ellipticity) $E = L/W$, area $A = \pi * \frac{L}{2} * \frac{W}{2}$, perimeter $P = 2\pi \frac{\sqrt{L^2+W^2}}{2}$, surface area $S = 4\pi \left(\left(\frac{W}{2}\right)^2 + (L-W) * 2\pi \frac{W}{2}\right)$, and volume $V = \frac{4}{3}\pi * \frac{L}{2} * \left(\frac{W}{2}\right)^2$. A total of 200 spermatozoa across 3 males were measured for morphology.

3.3.3.3 Automated sperm count and motility

(i) CASA detection parameters

The computer-assisted sperm analysis (CASA) system consisted of an Olympus BX53/CoolLED pE-300W fluorescent phase-contrast microscope (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 AndroVision®, version 1.1 software (Minitüb GmbH, Tiefenbach, Germany). Videos of spermatozoa were recorded at 10 X final magnification. The AndroVision® software was calibrated to detect barramundi spermatozoa based on manual sperm morphology measurements (Table 3.1) 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; the *field of view*, 555.12 µm x 555.12 µm.

Table 3.1 Characterization of sperm quality parameters in barramundi (*Lates calcarifer*) broodstock.

Parameter	Mean ± SEM (*n*)	Range (min-max)
Milt characteristics		
Volume (µL)	44.2 ± 8.0 (10)	9.3 - 91.8
Concentration (x 10^9 sperm/mL)	15.9 ± 2.0 (10)	2.9 - 33.3
Total count (x 10^6 sperm)	576.4 ± 146.7 (10)	126.8 - 1334.7
Sperm morphology		
Head length (µm)	2.44 ± 0.03 (200 sp)	1.71 - 3.83
Head width (µm)	2.23 ± 0.03 (200 sp)	1.48 - 3.21
Head ratio (length/width)	1.11 ± 0.01 (200 sp)	0.65 - 1.76
Head area (µm^2)	4.31 ± 0.07 (200 sp)	2.22 - 7.34
Head perimeter (µm)	14.75 ± 0.12 (200 sp)	10.75 - 19.59
Head surface area (µm^2)	17.23 ± 0.29 (200 sp)	8.90 - 29.36
Head volume (µm^3)	6.60 ± 0.17 (200 sp)	2.19 - 15.29
Tail length (µm)	30.99 ± 0.49 (200 sp)	10.04 - 44.95
Total length (µm)	33.43 ± 0.49 (200 sp)	12.67 - 47.61
Sperm motility[†]		
Total motility (%)	24.5 ± 4.4 (8)	11.3 - 52.4
Progressive motility (%)	9.4 ± 1.9 (8)	1.7 - 20.3
Slow motility (%)	15.1 ± 3.7 (8)	3.7 - 32.1
Medium motility (%)	5.7 ± 0.8 (8)	1.7 - 7.9
Fast motility (%)	3.7 ± 1.4 (8)	0.0 - 12.3
VCL (µm/s)	55.6 ± 8.2 (8)	20.8 - 98.6
VSL (µm/s)	36.7 ± 6.9 (8)	8.3 - 70.7
VAP (µm/s)	44.7 ± 8.2 (8)	10.6 - 86.8
LIN (%)	49.8 ± 3.9 (8)	34.0 - 68.2
STR (%)	72.3 ± 2.6 (8)	61.1 - 84.0
WOB (%)	63.6 ± 3.7 (8)	46.5 - 78.8
ALH (µm)	0.46 ± 0.02 (8)	0.33 - 0.54
BCF (Hz)	8.6 ± 1.3 (8)	4.0 - 14.0
Sperm integrity		
Dead (%)	33.9 ± 2.9 (10)	18.2 - 49.9
DNA-damaged (%)	43.5 ± 6.0 (9)	16.3 - 76.5

Data are displayed as mean ± standard error (SEM)*; n*, number of fish; sp, number of spermatozoa; 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); curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), straightness of the average path (STR), linearity of the curvilinear path (LIN), wobble (WOB) and beat-cross frequency (BCF).[†]see Appendix A for sperm motility data per fish.

(ii) CASA automated calculation of sperm concentration

The automated calculation of sperm concentration was determined using the AndroVision® Sperm Motility module, where the number of spermatozoa per field of view (*N*) was divided by the fluid volume contained in each field of view (*V*), and multiplied by the dilution factor,

That is, $$[c] = \frac{N}{V} \times dilution\ factor$$

$$where\ V = Leja\ chamber\ depth \times field\ width \times field\ height \quad \text{(Eq 1)}$$

Leja 4-chamber linear-flow slides (Leja Products BV, Nieuw Vennep, Amsterdam, Netherlands) used with this system were 20 µm deep, resulting in a volume (*V*) of 6.16 x 10^{-6} mL.

(iii) CASA motility evaluation

Upon collection, sperm samples were diluted with the artificial extender MRS400 at a dilution ratio of 1:20. Once homogenized, the sample was further diluted 1:50 in salt water (final dilution 1:1000) to activate sperm motility. Then 3 µL of homogenized activated spermatozoa were loaded into a 30 ˚C pre-warmed Leja slide chamber and analyzed by the AndroVision® *Sperm Motility* module. Videos were recorded 15 s after each sperm sample was activated in salt water at a frame rate of 60 fps for 1 s. The analysis of motility was performed in triplicate for each fish. The following sperm motility parameters were evaluated: 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), amplitude of lateral head displacement (ALH; µm), straightness of the average path (STR; % of VSL/VAP), linearity of the curvilinear path (LIN; % of VSL/VCL), wobble (WOB; % of VAP/VCL) and beat-cross frequency (BCF; Hz) as previously described in Mortimer (2000).

3.3.3.4 CASA threshold settings for sperm motility

Spermatozoa were considered motile when VCL ≥ 15 µm/s, excluding immotile spermatozoa, influenced by capillary action within the Leja chamber. Progressive motility was defined as sperm with VCL ≥ 35 µm/s. Sperm motility subpopulations were classified based on the following VCL thresholds:

Slow motility (VCL ≥ 15 and < 35 μm/s), Medium motility (VCL ≥ 35 and < 100 μm/s) and Fast motility (VCL ≥ 100 μm/s).

3.3.4 CASA validation experimental design

3.3.4.1 Trial 1.1: Validation of sperm detection accuracy by CASA

Sperm count, as a measure of the accuracy of sperm head detection, was assessed automatically across 90 videos of barramundi spermatozoa captured by CASA. Automatic sperm counts were directly compared with manual counts using the same video. For manual counts, digital still images were captured by AndroVision® software before and after each video assessment. These images were imported into ImageJ software to permit manual identification and counting of barramundi spermatozoa using the multipoint tool. A total of 12,457 spermatozoa from 10 barramundi were identified and counted using both methods to validate the sperm detection accuracy of CASA.

3.3.4.2 Trial 1.2: Validation of automated calculation of sperm concentration by CASA

For each fish (n = 10), spermatozoa were automatically counted and averaged across seven distinct fields of view at each of five dilutions of spermatozoa in salt water (1:250, 1:500, 1:1000, 1:2500, and 1:5000). The assessment of each dilution was performed in triplicate. Sperm concentration was calculated automatically using the algorithm described earlier (Eq 1). The same sample dilutions were then compared to sperm concentrations obtained with a haemocytometer (WHO, 2010). Manual counts were performed in triplicate (for each of the 2 chambers) using an improved Neubauer haemocytometer. Due to the relatively high concentration of barramundi spermatozoa observed on the haemocytometer slide at these dilutions, the 5-diagonal small sub-squares (R; 0.004 mm^3 each) within the large central square (W) were used to count sperm according to the Neubauer ruling (WHO, 2010). The manual calculation of sperm concentration was determined using the mean number of spermatozoa per 5 small squares across both chambers (N), multiplied by the square multiplication factor 50,000 for (R), and multiplied by the dilution factor.

$$[c] = N \times 50{,}000 \times dilution\ factor \qquad \text{(Eq 2)}$$

3.3.4.3 Trial 1.3: Determination of the minimum number of fields required for accurate calculation of sperm concentration by CASA

For each fish (n = 10), an average sperm concentration was calculated automatically with CASA by using counts averaged across each of 1–7 fields of view using sperm samples diluted at 1:1000 in saltwater. The same samples were then subjected to the manual calculation of sperm concentration by an improved Neubauer haemocytometer as described above (WHO, 2010). Both automated and manual counts were performed in triplicate. To assess the accuracy of counts, results were expressed as the mean coefficient of variation of sperm concentration between replicates for both CASA and haemocytometer methods.

3.3.4.4 Trial 1.4: Determination of the effect of motility on the accuracy of sperm concentration by CASA

Sperm concentration was assessed with CASA across 60 videos of barramundi spermatozoa. In order to compare the accuracy of sperm concentration on motile *vs.* immotile cells, spermatozoa for each fish (n = 10) were assessed in triplicate upon activation in salt water (two-step dilution to a final ratio of 1:1000 as described in the CASA motility section) by CASA. For each replicate, a video recording of motile spermatozoa (captured 15 s after saltwater activation) was compared to another video recording of non-motile spermatozoa from the same chamber (captured after cessation of motile activity ~1 min after saltwater activation).

3.3.4.5 Trial 1.5: Preliminary characterization of barramundi spermatozoa motility using CASA

For each fish (n = 10), sperm motility was measured 15 s after activation with salt water at a final dilution ratio of 1:1000. A total of 12,925 spermatozoa were assessed across the 10 fish; however, only spermatozoa with a VCL $\geq$ 15 μm/s were included in subsequent analyses. Fish (n = 2) were excluded from the characterization of sperm velocities due to an insufficient number of motile spermatozoa after the exclusion of immotile sperm influenced by capillary action.

3.3.5 Viability assay

The viability of spermatozoa was assessed by measuring plasma membrane integrity using Hoechst 33342 and propidium iodide (PI), according to a modified version of Cummings et al. (2004). Briefly, for each fish (n = 10), the concentration of each sperm sample was standardized to 100 x 10^6 sperm/mL in MRS400. An aliquot from each sample was used to generate an unstained control (U1), heat-treated Hoechst single-stained positive control (P1), heat-treated PI/Hoechst double-stained positive control (P2), and test sample. Positive controls were incubated at 70 °C for 5 min to induce perforation of the sperm cell membrane and subsequent complete PI staining. Positive controls and test samples were each incubated at room temperature for 30 min in 10 µg/mL Hoechst 33342 in MRS400 and further incubated for 7 min in the dark in 10 µg/mL PI. A dilution curve was prepared by diluting the positive control (100% membrane ruptured red nucleated cells) at fixed ratios with its corresponding test sample (undamaged blue nucleated cells; 0:100, 25:75, 50:50, 75:25, and 100:0%). The percentage of dead spermatozoa detected by flow cytometry was compared to the expected ratio of damaged/undamaged cells for each dilution. Test samples were then individually analyzed by flow cytometry. The specificity of sperm staining was validated visually using an Olympus BX53/CoolLED pE-300W fluorescent microscope at 20 X final magnification (Figure 3.1f).

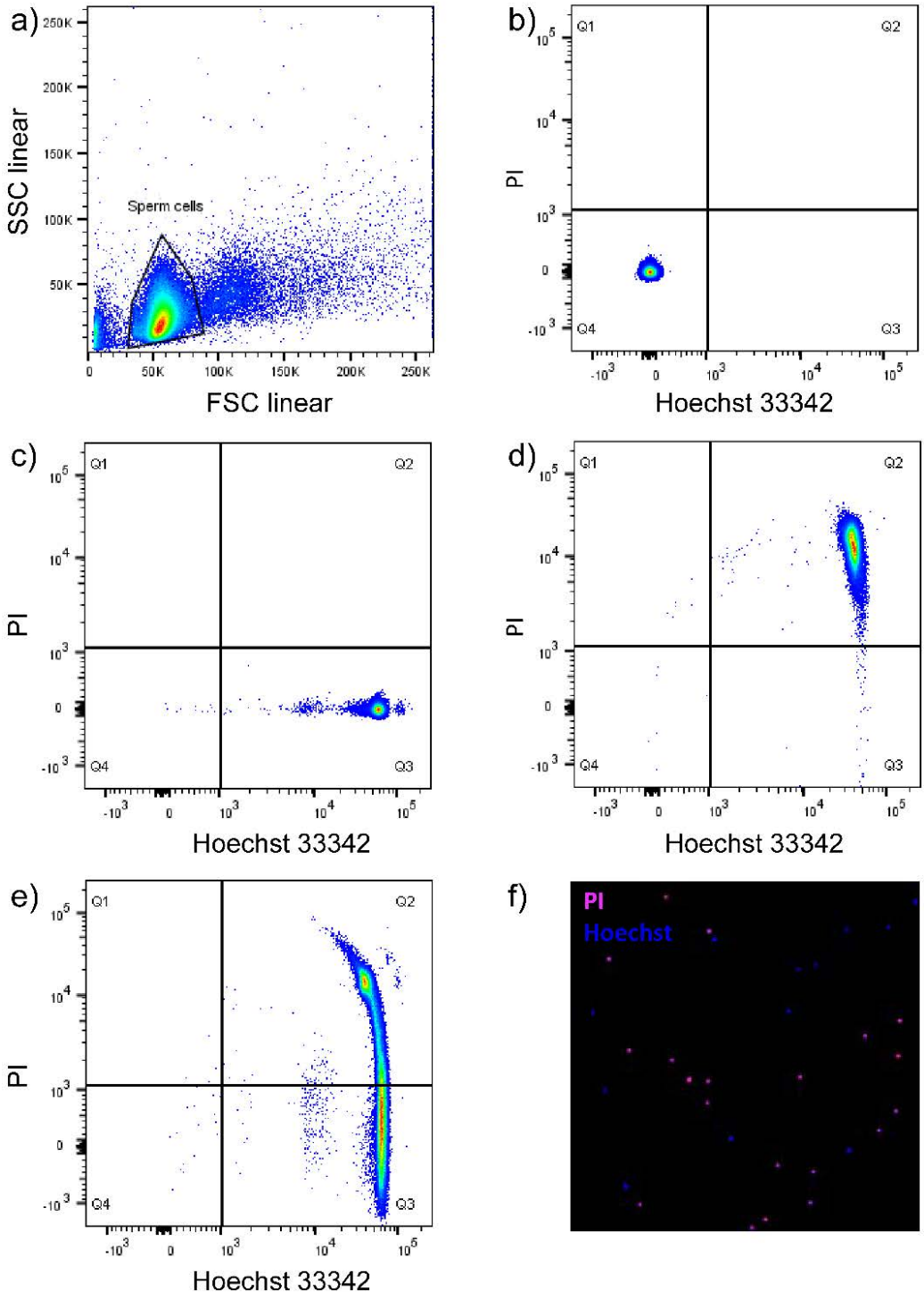

Figure 3.1 Validation of sperm viability assay and flow cytometry for barramundi (*Lates calcarifer*) spermatozoa subjected to different staining treatments for PI and Hoechst 33342. a) SSC/FSC scatter plot showing the gating used for unlabeled spermatozoa, U1; PI/Hoechst plot for b) unlabeled control, U1; c) heat-treated Hoechst positive control, P1; d) heat-treated PI/Hoechst positive control, P2; e) test sample showing dead sperm subpopulation in Q2; f) 20 X microscopic validation of viability assay showing membrane-damaged/dead (pink; PI+/Hoechst+) and intact/live (blue; Hoechst+) spermatozoa. SSC, side scatter; FSC, forward scatter; PI, propidium iodide; Q1, PI+/Hoechst-; Q2, PI+/Hoechst+; Q3, PI-/Hoechst+; Q4, PI-/Hoechst-.

3.3.6 DNA fragmentation assay

The presence of DNA fragmentation in spermatozoa was assessed using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method (In Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Mannheim, Germany), modified according to Peña et al. (2019) and Figueroa et al. (2013). Briefly, for each fish (n = 10), the concentration of each sperm sample was standardized to 100 x 10^6 sperm/mL in MRS400. An aliquot from each was used to generate an unlabelled control (U1), unlabelled 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 a DNase-treated FITC-positive control with PI (P2). Next, controls and test samples were centrifuged at 320 g for 5 min and each pellet fixed in fresh 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4) for 1 h at room temperature. The sample was washed in PBS at 320 g for 5 min and the pellet 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 then washed in PBS at 320 g for 5 min. Positive controls were re-suspended 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 re-suspended in the appropriate solution: unlabelled controls (U1 and U2) in 50 µL PBS, negative controls (N1 and N2) in 50 µL TUNEL labelling solution without enzyme, and positive controls (P1 and P2) and each biological sample in 50 µL of TUNEL reaction mixture containing enzyme. All samples were incubated 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.

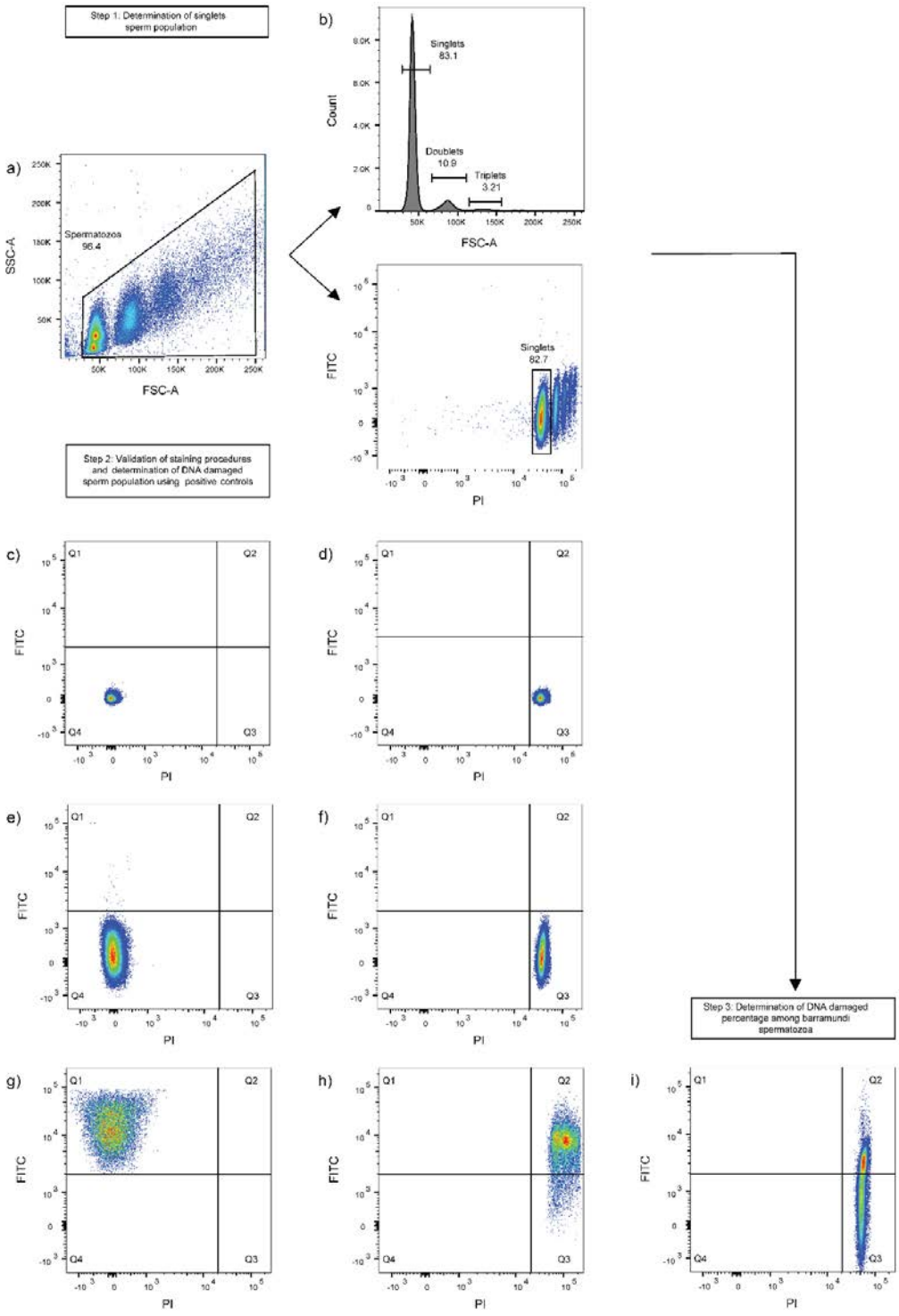

Figure 3.2 Validation of sperm DNA integrity assay using flow cytometry for barramundi (*Lates calcarifer*) spermatozoa subjected to different staining treatments for FITC (TUNEL) and PI. a) SSC/FSC scatter plot showing the gating used to exclude debris from the unlabeled sperm population, U1; b) Count/FSC plot for unlabeled control, U1, showing single sperm peak and FITC/PI plot for unlabeled control with PI, U2, showing gating for single cells; FITC/PI plot for c) unlabeled control, U1; d) unlabeled control with PI, U2; e) negative control in label solution, N1; f) negative control in label solution with PI, N2; g) DNase-treated FITC positive control, P1; h) DNase-treated FITC positive control with PI, P2; i) test sample showing DNA-damaged sperm subpopulation in Q2. SSC, side scatter; FSC, forward scatter; FITC, fluorescein isothiocyanate; PI, propidium iodide; Q1, FITC+/PI-; Q2, FITC+/PI+; Q3, FITC-/PI+; Q4, FITC-/PI-.

3.3.7 Flow cytometry

Sperm samples labeled to detect cell viability or DNA fragmentation were measured separately using a FACS Canto™ II flow cytometer (8-color; blue/red/violet lasers; serial number: V33896202614; manufactured May 2016; BD Biosciences, San Jose, CA, USA). Before each analysis, the three lasers were calibrated using control quality beads (Lot: 7130918, BD FACSDiva™ CS&T Research Beads, San Jose, CA, USA). 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 = 225 V, FITC filter = 495 V and PI filter = 537 V. The sperm population was identified using side and forward scatter profiles from unstained samples (Figure 3.1a and 3.2a-b). Control samples were used to define the different subpopulations: Q1 assay-specific stain only (-;+), Q2: dual stains (+;+ damage cells), Q3: nuclear counterstain only (+;-), Q4: no stain (-;-) (Figure 3.1b-d and 3.2c-h). The flow cytometer was set to analyze 100,000 cells per sample. The different subpopulations were expressed as the percentage of the total number of events identified as spermatozoa.

3.3.8 Statistical analysis

Statistical analyses were performed using custom scripts in RStudio V1.0.153 (RStudio Inc., Boston, MA, USA). Dependence and relationships between variables were analyzed using Pearson's method by linear regression (r). Differences were considered significant at $P < 0.05$. The intraclass correlation coefficient (ICC) was used to assess agreement between methods based on the 95% confidence interval of the ICC, with values greater than 0.9 indicating high consistency between measurements. The coefficient of variation (CV) was defined as the standard deviation divided by the mean and expressed as a percentage. Data are displayed as mean ± standard error (SEM).

3.4 Results

3.4.1 Milt assessment

The characteristics of milt samples collected were highly variable among males. Across the 10 fish, milt volume ranged from 9.3 to 91.8 µL, sperm concentration varied from 2.9 to 33.3 x 10^9 sperm/mL, and total sperm count ranged from 126.8 to 1334.7 x 10^6 sperm (Table 3.1).

3.4.2 Sperm morphology

Barramundi spermatozoa are uniflagellate and appear to consist of three morphological components: a small elliptical head approximately 2.4 µm long (head ratio close to 1.1), a short transitional region (midpiece, size not measured), and a single long flagellum of approximately 31 µm (93% of the total sperm length; Table 3.1; Figure 3.3). The presence of a defined acrosome (i.e., head cap) was not observed in barramundi spermatozoa. Other calculated parameters, including head area (µm^2), head perimeter (µm), head surface area (µm^2), and head volume (µm^3), are shown in Table 3.1.

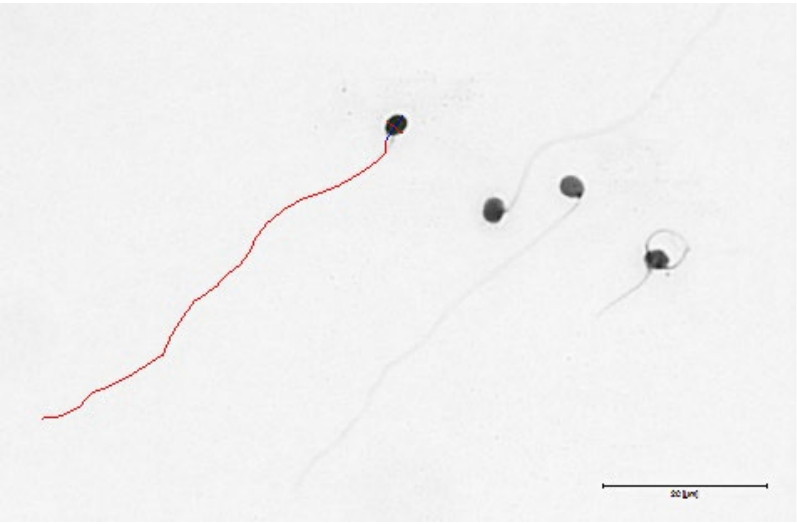

Figure 3.3 SpermBlue-stained spermatozoa of barramundi (*Lates calcarifer*) visualized by bright field illumination at 50 X magnification; Morphometric measures are indicated in red (head width and tail length) and blue (head length). Scale bar = 20 µm.

3.4.3 Trial 1.1: Validation of sperm detection accuracy by CASA

Sperm counts generated from each video using the CASA automatic detection algorithm were highly correlated to the manual counts obtained from the haemocytometer (r = 0.99, P < 0.001). This demonstrates, with the thresholds described, that CASA can accurately distinguish barramundi spermatozoa from other contaminants or debris within the samples (Figure 3.4).

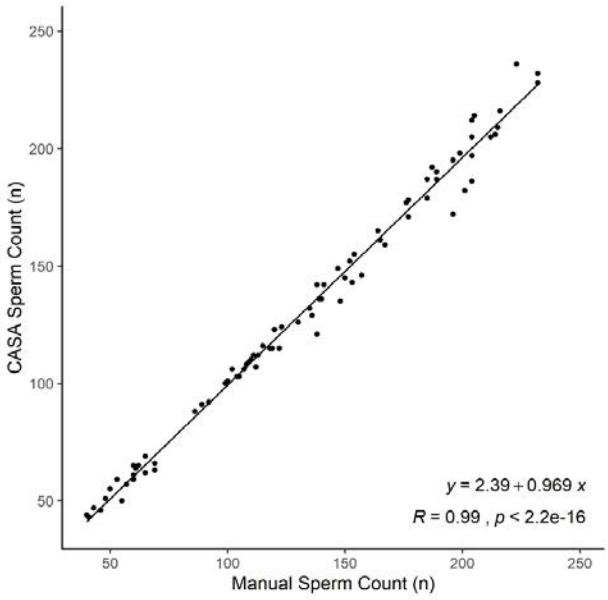

Figure 3.4 Relationship between automated computer-assisted sperm analysis (CASA) and manual count for spermatozoa from barramundi (*Lates calcarifer*).

3.4.4 Trial 1.2: Validation of automated calculation of sperm concentration by CASA

A high coefficient of correlation was observed for each dilution ratio, including 1:250 (r = 0.78, P < 0.001), 1:500 (r = 0.72, P < 0.001), 1:2500 (r = 0.72, P < 0.001), and 1:5000 (r = 0.77, P < 0.001), and indicates a strong correlation between both methods across a broad range of dilution factors (Figure 3.5). The concordance between sperm concentration determined by CASA and manual counts was the highest when a 1:1000 dilution ratio was used (r = 0.88, P < 0.001; Figure 3.5c). The intraclass correlation coefficient also had the highest precision at 1:1000 dilution (ICC = 0.99; Figure 3.5h). Thus, a sperm dilution ratio of 1:1000 would achieve the most reliable barramundi sperm concentration with CASA.

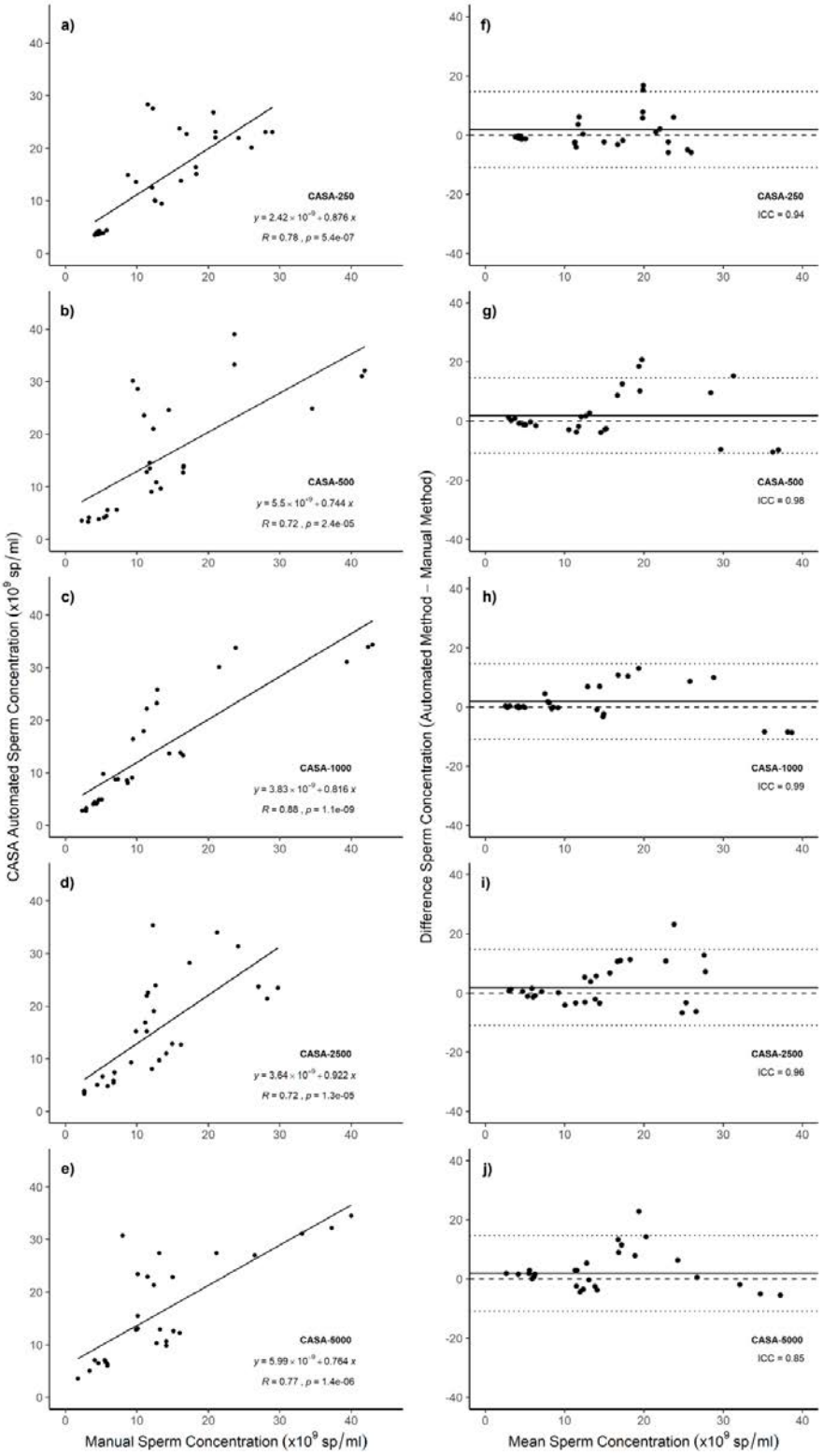

Figure 3.5 Relationship between mean automated CASA and manual haemocytometer concentration for barramundi (*Lates calcarifer*) spermatozoa at a) 1:250, b) 1:500, c) 1:1000, d) 1:2500, and e) 1:5000 dilution. Overall limits of agreement showing mean (solid line) ± SD (dotted lines) difference in sperm concentration measured by CASA and haemocytometer at f) 1:250, g) 1:500, h) 1:1000, i) 1:2500, and j) 1:5000 dilution. Mean sperm concentration across 7 fields (CASA) and 2 chambers (haemocytometer) from n = 10 barramundi conducted in triplicate. The correlation coefficient (r) and the intraclass correlation coefficient (ICC) are displayed for each dilution.

3.4.5 Trial 1.3: Determination of the minimum number of fields required for accurate calculation of sperm concentration by CASA

The mean coefficient of variation between the three replicates was 8.0% when sperm concentration was calculated by manual haemocytometer across all 10 fish (Table 3.2). The mean coefficient of variation between the three replicates CASA sperm counts reduced from 14.0 to 6.2% when the number of fields was increased from 1 to 7. Thus, sperm concentration calculated with greater or equal to 4 fields of view by CASA (CASA-4 = 7.5% CV) will have similar or improved precision than the 'gold-standard' haemocytometer method (Table 3.2). The optimized CASA count method with 4 fields was applied to the 10 male barramundi test samples, yielding a mean sperm concentration of $15.1 \pm 3.6 \times 10^9$ sperm/mL (Table 3.1).

Table 3.2 Coefficient of variation between manual haemocytometer and automated computer-assisted sperm analysis of sperm concentration in barramundi (*Lates calcarifer*) at 1:1000 dilution. Data were calculated using mean values derived from two chambers (haemocytometer; Neubauer improved) and mean values from 1 to 7 fields (CASA-1 to 7) across $n = 10$ barramundi each assessed in triplicate.

Male number	Haemocytometer	CASA-1	CASA-2	CASA-3	CASA-4	CASA-5	CASA-6	CASA-7
1	5.2	7.4	8.3	4.3	1.5	2.0	3.3	4.0
2	4.4	10.6	10.3	5.7	5.1	7.4	5.3	5.5
3	10.2	5.7	9.5	8.8	8.6	8.1	7.1	6.0
4	7.3	4.8	4.7	3.8	6.6	6.3	6.3	5.9
5	16.8	4.1	0.8	5.5	6.6	6.3	6.2	6.3
6	6.7	24.8	21.3	15.5	11.2	8.8	9.1	8.0
7	4.6	13.1	7.7	4.2	4.7	5.5	5.2	5.4
8	6.9	28.4	18.1	12.5	7.2	10.2	8.4	6.3
9	6.6	9.4	4.9	4.5	5.7	2.9	1.0	2.3
10	11.2	32.2	16.4	22.2	17.9	16.5	11.8	11.7
Mean	8.0	14.0	10.2	8.7	7.5	7.4	6.4	6.2

3.4.6 Trial 1.4: Determination of the effect of motility on the accuracy of sperm concentration by CASA

Sperm concentration determined by CASA was highly correlated when using motile or immotile cells (r = 0.98, P < 0.001; Figure 3.6). This demonstrates that motility does not negatively affect the ability of CASA to quantify barramundi spermatozoa accurately and that sperm counts and motility estimates can be collected simultaneously from samples.

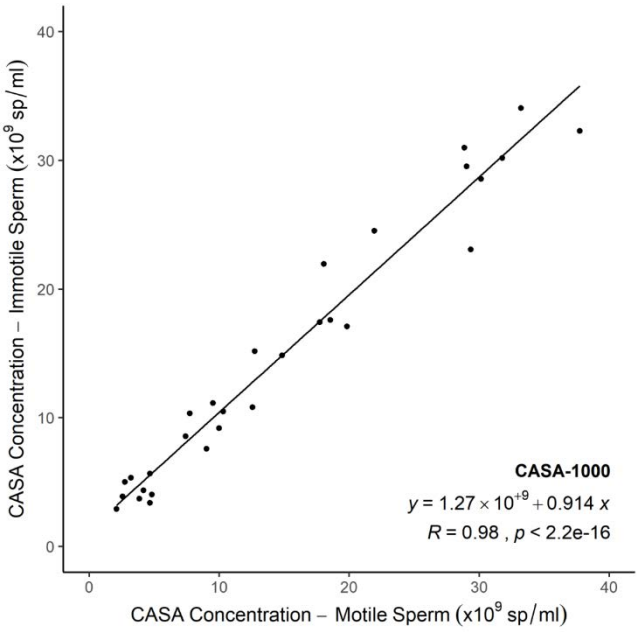

Figure 3.6 Relationship between sperm concentration in motile and immotile barramundi (*Lates calcarifer*) spermatozoa using computer-assisted sperm analysis (CASA) at 1:1000 dilution. n = 10 barramundi conducted in triplicate.

3.4.7 Trial 1.5: Preliminary characterization of barramundi spermatozoa motility using CASA

Sperm motility was found to be low, with an average of 24.5 ± 4.8%, and highly variable between males, ranging from 11.3–52.4% (Table 3.1; Appendix A). Spermatozoa that were considered to have progressive motility represented on average less than 10% of samples assessed (Table 3.1). Specifically, spermatozoa with slow motility were most commonly observed (15.1 ± 3.7%), followed by medium motility (5.7 ± 0.8%) and fast motility (3.7 ± 1.4%). The high proportion of spermatozoa with slow

motility is corroborated by the generally low mean VCL, VAP, and VSL speeds of 55.6 ± 8.2 µm/s, 36.7 ± 6.9 µm/s, and 44.7 ± 8.2 µm/s, respectively (Table 3.1). Despite the considerable variation in the proportion of motile spermatozoa between males (Appendix A), swimming trajectory was observed to be relatively steady, with a curvilinear motion (LIN: 49.8 ± 3.9%) that has limited lateral deviation from the main path (STR: 72.3 ± 2.6, WOB: 63.6 ± 3.7% and ALH: 0.46 ± 0.02 µm). Sperm motility characteristics for each male are described in Appendix A.

3.4.8 Viability assay

A single-cell population of barramundi spermatozoa could be identified and was isolated from debris, doublets, and triplets present in the sample (contamination: 14.9 ± 6.2% of 100,000 events) on the SSC x FCS scatterplot of the flow cytometer (Figure 3.1a). Heat treatment of the positive control (P2) disrupted 99.9 ± 0.1% of sperm cell membranes (i.e., induced cell death; Figure 3.1d) without altering their forward and side-scatter properties or introducing additional debris within the sample (88.5 ± 0.9% of 100,000 events identified as spermatozoa). All other controls (U1 and P1) were consistent with their predicted emission spectra (Figure 3.1b and c), with membrane-damaged/dead barramundi spermatozoa identified in pink alongside intact/live spermatozoa in blue (Figure 3.1f). Using a 5-point dilution curve, consisting of positive control and test samples mixed at different ratios, the assay was able to detect the proportion of viable cells with 98.4% accuracy compared to predicted values ($r = 0.98$; $P < 0.05$; Figure 3.7), demonstrating that dual-staining with Hoechst and PI can accurately identify cell death in barramundi spermatozoa. When applied to test samples, live and dead sperm populations could be separated into Q3 and Q2 quadrants, respectively (Figure 3.1e). Moreover, the mean proportion of dead spermatozoa across the 10 male barramundi was relatively high (33.9 ± 2.9%) but variable, ranging from 18.2 to 49.9% (Table 3.1).

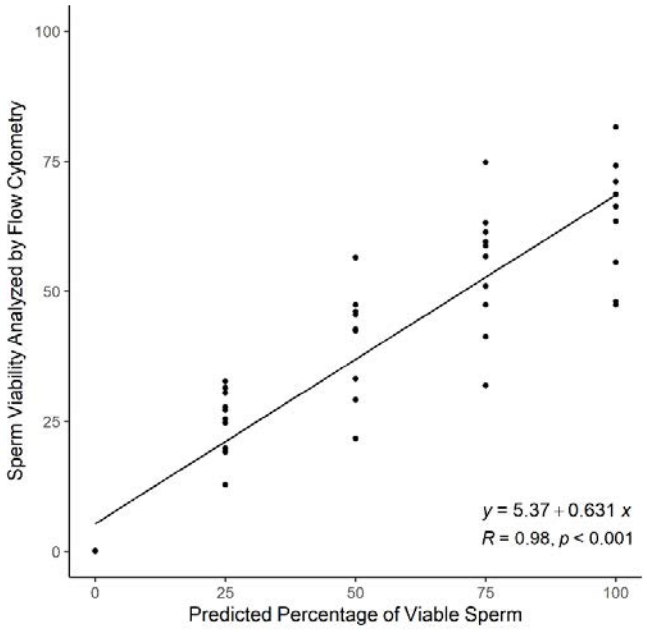

Figure 3.7 Relationship between membrane damage detected by viability assay *vs.* predicted damage based on a 5-point dilution curve of intact: damaged barramundi (*Lates calcarifer*) spermatozoa at 0:100, 25:75, 50:50, 75:25, and 100:0%. The analysis was performed in triplicate on spermatozoa from $n = 10$ fish.

3.4.9 DNA fragmentation assay

A two-step process was used to identify the population of single-cell sperm heads labeled by TUNEL assay. Firstly, the total sperm population was isolated from debris (74.7 ± 4.7% of 100,000 events) on the SSC x FCS scatterplot of the flow cytometer (Figure 3.2a). Secondly, the single-cell sperm population was identified and isolated from doublets and triplets (74.7 ± 1.1% of 100,000 events) on the count x FSC and FITC x PI scatterplots (Figure 3.2b). TUNEL labeling and particularly DNase treatment of positive controls altered the forward and side-scatter properties of spermatozoa. As such, gating the single-cell sperm population was performed using the count x FSC scatterplot for unstained controls or the FITC x PI scatterplot for stained controls and test samples. Observations for all controls (i.e., U1, U2, N1, N2, P1, and P2) were consistent with the predicted emission spectra for each stain (Figure 3.2c-h). Negative controls were used to set the horizontal threshold of the quadrant between the DNA-damaged and intact subpopulation (Figure 3.2e and f) and further validated using the positive controls (Figure 3.2g and h). PI stained controls (i.e., U2, N2, and P2) were used to set the vertical threshold of the quadrant between stained and unstained nucleated cells (Figure 3.2d, f, and h). When

applied to test samples, DNA-damaged spermatozoa could be detected in quadrant Q2 and the intact sperm population in Q3 (Figure 3.2i). Moreover, the mean proportion of DNA-damaged spermatozoa across the 10 male barramundi was high (43.5 ± 6.0%) and was highly variable across individuals, ranging from 16.3 to 76.5% (Table 3.1).

3.5 Discussion

In this study, we have optimized and validated several advanced tools to evaluate sperm function in barramundi. AndroVision® computer-assisted sperm analysis (CASA) using a sample dilution of 1:1000 captured over four or more fields of view, can accurately quantify barramundi spermatozoa while simultaneously performing sperm motility assessment. Moreover, both the cell viability and TUNEL assays described can accurately identify cell death and DNA damage, respectively, in barramundi spermatozoa.

The application of these tools to barramundi broodstock provides the first understanding of key sperm characteristics for the species. Barramundi have highly concentrated small ovoid uniflagellate spermatozoa, which were observed to demonstrate low yet highly variable motility. Furthermore, low cell viability and DNA integrity were observed. Ultimately, these tools will allow future investigation of the cause of variable fertilization rates and highly skewed paternity often seen in spawning events. The techniques optimized in the current study will also enable screening and selection of male broodstock based on fertility within commercial hatcheries before breeding.

Sperm concentration has been traditionally measured using a haemocytometer and is considered the ‘gold standard’ by WHO (Prathima et al., 2015). However, manual counts are time-consuming and prevent the rapid processing of sperm samples within routine commercial broodstock assessment workflows. Other methods, including micro-haematocrit, flow cytometry, cell counter, spectrophotometry, and CASA, are considered reliable alternatives for measurement of sperm concentration in humans, mammals (Brito et al., 2016), model fish species (zebrafish, *Danio rerio*;

Chen et al., 2017; fighting fish, *Betta splendens*; Montgomery et al., 2014; see review Yang and Tiersch, 2009), and several major aquaculture species (salmonids; Judycka et al., 2016; brook trout, *Salvelinus fontinalis*; Nynca and Ciereszko, 2009; European eel, *Anguilla anguilla*; Sorensen et al., 2013). CASA is an attractive alternative due to its comprehensive set of functionalities, allowing simultaneous assessment of sperm concentration and motility, among others. With the increasing number of CASA software platforms that are available, coupled with the large variation in hardware, equipment, and experimental design, concern has been raised about the lack of a standardized procedure for CASA (Ehlers et al., 2011; Gallego et al., 2013a; Horváth et al., 2012; Rosenthal et al., 2010; Wilson-Leedy and Ingermann, 2007). Parameters, such as frame rate, recording time, and recording quality, have been observed to dramatically affect motility values with each fish species assessed (Boryshpolets et al., 2013; Gallego et al., 2013a). To address these concerns, we assessed a series of key sperm quality parameters in barramundi for their reliability and repeatability when obtained by CASA. In the present study, we confirmed CASA was highly accurate in the quantification of barramundi spermatozoa when compared to manual methods ($r = 0.99$, $P < 0.001$), validating the automatic AndroVision® detection profile developed. Discrepancies in motility can occur when using the same program settings for different fish species if species-specific CASA parameters are not validated (Boryshpolets et al., 2013). A similar species-specific approach was also performed to validate the automatic CASA detection profile for European eels (Gallego et al., 2013a), which, along with our results, provides a robust framework for the development of CASA profiles for other aquaculture species.

Sperm concentrations measured by CASA were highly consistent with those derived from manual haemocytometer, with the strongest correlation ($r = 0.87$, $P < 0.001$), and best precision (ICC = 0.99; 95% confidence interval) at 1:1000 dilution, however across all dilutions the correlation was considered to be acceptable ($r \geq 0.72$, ICC ≥ 0.85). In previous studies of other fish species, milt dilution had also been found to have little effect on the estimation of sperm concentration, with the greatest consideration being to dilute spermatozoa sufficiently to ensure swimming is not impeded (i.e., avoid sperm collision; Sorensen et al., 2013; Wilson-Leedy and Ingermann, 2007). Furthermore, estimation of sperm concentration was more precise with an increasing number of microscope fields recorded,

outperforming the haemocytometer (CV = 8.0%) when at least four fields were recorded. A similar trend is observed in livestock studies (reviewed in Verstegen et al., 2002), but differs to coefficients of variation reported by Sorensen et al. (2013) in which CV increased with an increasing number of recorded fields (1 *vs*. 3 fields; CV = 17.9% *vs*. 28.4%, respectively). Lastly, CASA-estimated sperm concentration was equally reliable using either motile or immotile spermatozoa ($r = 0.99$, $P < 0.001$).

Artefactual motility of immotile sperm caused by capillary action was measured, and a minimum VCL threshold of ≥ 15 μm/s was required to classify sperm as motile. This is similar to threshold values reported for European eel (VCL > 10 μm/s; ISAS v1; Gallego et al., 2013a), but lower than the default cut off value (VCL > 25 μm/s) used on the ImageJ CASA plug-in for several fish species (Boryshpolets et al., 2013; Wilson-Leedy and Ingermann, 2007). Using either threshold published for European eel or from the default settings in ImageJ would under- or overestimate the motility of barramundi spermatozoa. As such, determining CASA-specific exclusion thresholds for immotile drifting sperm from each species is essential to the accurate estimation of sperm motility.

Viability and DNA integrity assays were successfully optimized for barramundi. Furthermore, live: dead dilution curves confirmed the accuracy (98.4%) of Hoechst 33342 and PI dyes to detect dead spermatozoa, with staining specificity confirmed by microscopy. Similar accuracy has been reported with Hoechst 33342/PI stained spermatozoa in sheep (*Ovis aries*; Yániz et al., 2013), rhesus monkeys (*Macaca mulatta*; Cai et al., 2005), and boars (*Sus scrofa domesticus*; Garner et al., 1996), while, SYBR 14/PI was used to quantifying viability in several fish species (Cabrita et al., 2011; Dietrich et al., 2014; Figueroa et al., 2016b, 2016a, 2015, 2013; Flajšhans et al., 2004; Pérez-Cerezales et al., 2010; Segovia et al., 2000). Triple staining using Hoechst/SYBR 14/PI for mammalian spermatozoa (Cai et al., 2005; Garner et al., 1996) demonstrated SYBR 14 and Hoechst could be used interchangeably.

The use of the TUNEL assay to assess the DNA integrity of barramundi spermatozoa was validated with a series of positive and negative controls. DNA integrity assessment in fish is limited to a small number of studies on cryopreservation in Atlantic salmon (Figueroa et al., 2015, 2013), carp (*Cyprinus*

carpio; Öğretmen et al., 2015), European sea bass (*Dicentrarchus labrax;* Zilli et al., 2003), gilthead seabream (*Sparus aurata*; Cabrita et al., 2005a; Cartón-García et al., 2013), and rainbow trout (*Oncorhynchus mykiss*; Cabrita et al., 2005b; Figueroa et al., 2013; Pérez-Cerezales et al., 2010) and has primarily utilized an alternate DNA damage assay (referred to as the comet assay). While commonly used, the processing and assessment of samples with the comet assay is manually complex and time-consuming, relying upon quantification of the intensity, brightness, and length of the comet tail (DNA breaks migrating through agarose gel at different rates by electrophoresis) relative to the head to determine DNA damage using fluorescent microscopy. Instead, analysis of DNA integrity with the TUNEL assay can leverage the high-throughput screening capacity of flow cytometry to decrease assessment time and increase the accuracy of quantification by being able to assess approximately 100,000 spermatozoa in 2 min.

In this study, we describe the characteristics of spermatozoa and milt parameters for the first time in barramundi. The total milt volume typically collected was small on average (mean: 44.2 ± 8.0 µL), with only three males having a volume greater than 50 µL. This is in contrast to Leung (1987) and Palmer et al. (1993), where several milliliters of milt were collected from ripe wild-caught males during the spawning season. The difference in these volumes may indicate that the male broodstock used in our study were not in peak reproductive condition at the time of collection. Several factors could explain this discrepancy. Firstly, the gonadosomatic index of captive-bred barramundi is known to be lower than that of wild-caught individuals (Gamage, 2001; Moore and Reynold, 1982), indicating that the degree of functional maturation may also be lower under captive conditions. Secondly, in our study, milt samples were collected immediately before injection of luteinizing hormone-releasing hormone analog (LHRHa), which is typically required to induce milt hydration and spawning in captivity. It is unknown whether the assessment of sperm quality before injection accurately reflects the quality of barramundi spermatozoa released during spawning and warrants further investigation (Jerry, 2013; Moore, 1979).

The concentration of spermatozoa varied greatly between male barramundi in our study (11-fold difference between the highest and lowest concentration collected), with four male broodstock exceeding 30 x 10^9 sperm/mL, while the remaining individuals had less than 13 x 10^9 sperm/mL. However, no previous data on sperm concentration exists for barramundi for direct comparison. The variation observed in our study is similar to that observed in other marine teleosts, such as European sea bass (10–40 x 10^9 sperm/mL; Villani and Catena, 1991), and Atlantic halibut, *Hippoglossus hippoglossus* (20–60 x 10^9 sperm/mL; Tvedt et al., 2001). Furthermore, it has been suggested that sperm concentration is not a reliable indicator of natural spawning success in teleosts but more critical for artificial fertilization success (see review Rurangwa et al., 2004). The relationship between sperm concentration and skewed paternity after spawning remains to be determined for barramundi.

The morphology of barramundi spermatozoa described in our study is similar to that documented using a histological section of a barramundi spermatozoon by Leung (in Jamieson and Leung, 1991). Barramundi spermatozoa show simple sperm morphology, with a single uniflagellate tail, a poorly defined midpiece, and an ovoid head without an acrosome. This general morphology is consistent with many other teleosts, including other Perciforms (common Pandora, *Pagellus erythrine*; Maricchiolo et al., 2004), Esocidae (the Northern pike, *Esox lucius*; Alavi et al., 2009), Pleuronectidae (the Atlantic halibut; Alavi et al., 2011), and Cyprinidae (common carp; Billard et al., 1995). Head length of 2.44 µm was larger than that observed in other fish species (1.30–1.84 µm), except for spermatozoa from common carp (2.5 µm wide and 3.3 µm long; Billard et al., 1995).

As is characteristic of marine fish, the motility of barramundi spermatozoa was initiated by contact with salt water. However, the overall motility of the sample shortly after activation was low (total motility 24.5 ± 4.8%). Furthermore, the majority of motile spermatozoa had slow motility (15.1 ± 4.0%), with only 9.4 ± 2.0% showing progressive motility. The general lack of motility of barramundi spermatozoa observed in this study contrasts with studies, including Eurasian perch (*Perca fluviatilis*) and Atlantic halibut, which demonstrated 95% total motility when milt was obtained by manual stripping (Alavi et al., 2011a; Shaliutina et al., 2012). However, low sperm motility has also been observed in studies that

rely on gonadal cannulation for the collection of milt. In Japanese eel (*Anguilla japonica*), spermatozoa obtained from cannulation of the gonad showed low motility (~1.4%), while stripped spermatozoa demonstrated ~45% motility when activated (Ohta et al., 1997). Likewise, in turbot, gonadal cannulation of spermatozoa from the anterior, median, and posterior gonad were significantly lower (ranging from 40 to 55%) than spermatozoa collected by stripping (~95%; Suquet et al., 2000). In our study, spermatozoa could not be stripped from broodstock individuals; and is not frequently practiced in routine commercial practice due to its sporadic success and the potential risk of physical injury of highly valuable broodstock. The small milt volumes collected in our study and the low sperm motility observed suggest that most males may not have undergone milt hydration and, as such, spermatozoa obtained may not have been exposed sufficiently to maturation steroids to facilitate highly motile activity (see review Scott et al., 2010). In the future, the investigation of alternative methods for sperm collection is warranted, as the proportion of mature spermatozoa obtained by cannulation and/or the success of manual stripping may be improved with the provision of exogenous hormone treatment to promote sperm maturation and facilitate final milt hydration.

In this study, we used a modified Marine Ringer's solution to dilute sperm samples before further analysis, based on a previous study for barramundi by Palmer et al. (1993). It is known that sperm motility can also be affected by exposure to sub-optimal diluents (Morisawa and Suzuki, 1980). While designed to be a short-term holding media to keep spermatozoa dormant, the MRS400 (with optimized osmolality) used in this study may lack essential ions to facilitate complete activation of sperm motility after exposure to salt water. In particular, studies of several teleosts confirm the importance and different roles seminal plasma ions (i.e., Ca^{2+}, K^{+}, and Mg^{2+}) play in sperm motility (e.g., K^{+} inhibits motility of salmon spermatozoa; Alavi and Cosson, 2006; Dziewulska and Pilarska, 2018; Hamamah and Gatti, 1998; Judycka et al., 2016; Júnior et al., 2018; Morisawa and Suzuki, 1980; Vílchez et al., 2017; Wilson-Leedy et al., 2009; Yasui et al., 2012). To facilitate handling, storage, and further assessment of barramundi spermatozoa, further characterization of the ionic composition of barramundi seminal plasma would allow the formulation of an optimized diluent.

The proportion of dead barramundi spermatozoa (measured via membrane integrity) in this study was higher than expected and highly variable (mean: 33.9%; range: 18.2 to 49.9%). This was typically higher than the proportion of dead spermatozoa observed in freshwater species, including 9.5% for Atlantic salmon (*Salmo salar*; Figueroa et al., 2016b) and 1% for rainbow trout (De Baulny et al., 1997; Figueroa et al., 2013) but similar to that of other fish species including 25% for Senegalese sole (*Solea senegalensis*; Valcarce et al., 2016) and 23% for gilthead seabream (Cabrita et al., 2005a). Similarly, levels of DNA damage in barramundi spermatozoa in our study were also higher than expected (43.5 ± 6.0%), with a large variation between individuals sampled (16.3–76.5%). It is of note that levels of sperm viability and DNA damage observed in this study are similar to those observed in cryopreserved sperm of other teleosts post-thaw. In red snapper (*Lutjanus campechanus*), sperm viability after thawing ranged from 47 to 82% and from 44 to 75% for grey snapper (*Lutjanus griseus*; Riley, 2002). In European sea bass spermatozoa, DNA damage after thawing reached 38% (Zilli et al., 2003) and 49% in rainbow trout (Pérez-Cerezales et al., 2010). The high degree of DNA damage found in some males in this study may be associated with the high rates of dead spermatozoa in samples with lower viability. Dead spermatozoa are known to catalyze oxidation in viable spermatozoa, promote the production of free radicals, and cause further DNA fragmentation in viable spermatozoa (see review Figueroa et al., 2017). Additionally, cooling spermatozoa to 4 °C in humans (Wang et al., 1997) and rainbow trout (Pérez-Cerezales et al., 2009) has been found to damage the cell membrane and promote additional DNA damage. Further studies are necessary to determine the effect of short-term chilled storage on sperm function in barramundi to optimize sperm handling techniques.

3.6 Conclusion

This study provides the first quantitative description of barramundi sperm characteristics and comprehensive methods to assess sperm function. These tools provide a foundation to investigate further male reproductive biology and the development of sperm handling, storage, and assisted reproductive technology for the barramundi aquaculture industry, with the potential to be extended to other marine and warm-water species of commercial interest.

Chapter 4. Optimization of a non-activating medium for short-term storage of barramundi (*Lates calcarifer*) testicular milt

4.1 Abstract

Reliable storage of milt is a critical prerequisite to using advanced reproductive techniques for captive breeding of barramundi (*Lates calcarifer*), including artificial fertilization. Marine Ringer's solution (MRS) is a common medium used for storing teleost spermatozoa, including previously for barramundi. However, our trials showed that the use of MRS for storing testicular milt collected from captive-bred barramundi resulted in spermatozoa losing cell membrane integrity and undergoing cell lysis within 30 min. This suggested that MRS was not osmotically or ionically balanced to suit the cellular requirements of barramundi spermatozoa collected using testicular cannulation. Thus, the ionic and metabolite composition of seminal and blood plasma from captive-bred barramundi were characterized to refine the non-activating medium (NAM) composition. In particular, the effects of osmolality, pH, and Na^+ and K^+ concentrations on sperm viability and motility were examined to determine NAM's optimum composition to increase the longevity of barramundi spermatozoa in vitro. This was achieved through iteratively adapting the NAM ionic composition to mimic barramundi seminal plasma osmolality and ionic concentrations. Our data shows that increasing the medium osmolality from 260 to 400 mOsm/kg significantly improved sperm viability. The replacement of the buffering agent $NaHCO_3$ with HEPES significantly enhanced sperm motility and velocity. Testicular milt collected from barramundi by cannulation could be stored at 4 ℃ without significant loss of motility for up to 48 h when diluted with an optimized NAM containing 185 mM NaCl, 5 mM KCl, and 10 mM HEPES at pH 7.4. Furthermore, progressive sperm motility was retained for up to 72 h storage under these conditions. The optimized NAM developed in this study significantly extends the functional lifespan of barramundi spermatozoa *in vitro*, permitting the ongoing development of assisted reproductive technologies for this species.

4.2 Introduction

Efficient sperm handling and storage techniques are fundamental prerequisites to industrialized aquaculture using assisted reproductive technologies. The ability to temporarily store and distribute milt of superior barramundi broodstock coupled with artificial fertilization procedures will allow hatchery managers to achieve complete control over mate pairings and generate robust family-based selective breeding programs (Domingos et al., 2014, 2013; Robinson et al., 2010). Where artificial fertilization procedures have been incorporated into commercial practice for a particular aquaculture species, these procedures have proved pivotal to the industrialization of production (Gjedrem and Robinson, 2010; Janssen et al., 2015). However, the overall development and horizontal transfer of assisted reproductive technologies throughout the aquaculture industry has been hindered by differences in reproductive physiology and sperm biology across the wide range of species of commercial interest (Gjedrem and Robinson, 2010; Schulz et al., 2010). Therefore, precise knowledge of the sperm biology of each species needs to be obtained to allow the development of reliable species-specific sperm handling and storage protocols (Beirao et al., 2019; Cabrita et al., 2010).

Non-activating medium (NAM) aims to mimic the isotonic conditions and microenvironment of endogenous seminal plasma to maintain the quiescence and cellular integrity of spermatozoa during storage while also retaining a high activation potential (see review Beirao et al., 2019; Billard and Cosson, 1992). The imbalance between the physiochemical environment of spermatozoa and NAM can negatively alter sperm functionality and reduce its quality and fertilization potential upon activation (Alavi et al., 2004; Bozkurt et al., 2008; Dreanno et al., 1999a; Jing et al., 2009; Yang et al., 2006). Biochemical analysis of seminal plasma is the most direct approach to inform ionic composition and concentration for the NAM. Still, seminal plasma can be difficult to extract from milt due to its high viscosity and small volumes (< 100 µL) such as in Senegalese sole (*Solea senegalensis*; Cabrita et al., 2006; González-López et al., 2020). In contrast to mammals, the composition of seminal and blood plasma are similar in teleosts (Dietrich et al., 2014; Tan-Fermin et al., 1999). Thus, in some cases, blood

plasma analysis can be a suitable alternative to seminal plasma to help develop a NAM with appropriate osmolarity and ionic characteristics (Koya et al., 1993; Tan-Fermin et al., 1999).

In most teleosts, Na^+, K^+, Ca^{2+}, and Mg^{2+} are the main mineral compounds in seminal plasma and, at species-specific concentrations, maintain the quiescence of spermatozoa in the testes. Contact of spermatozoa with the external environment at ejaculation triggers Ca^{2+} influx that initiates motility (Alavi and Cosson, 2006; Krasznai et al., 2000). The Ca^{2+} influx can be triggered by exposure to several factors, including a low external concentration of K^+ (e.g., salmonids; Judycka et al., 2016), hypotonic shock (for many freshwater species; Billard, 1986), hypertonic shock (for many marine species; Billard, 1986), or the presence of egg-derived molecules (e.g., the herring *Clupea palasii*; Morisawa et al., 1992). In barramundi (*Lates calcarifer*), a euryhaline catadromous species that spawn in marine salinities, sperm motility is triggered by hypertonic shock (Leung, 1987; Palmer et al., 1993). This is consistent with the catadromous European (*Anguilla anguilla*) and Japanese eel (*Anguilla japonica*), as well as marine fish species (Cosson et al., 2008a, 2008b; Zilli et al., 2012).

Knowledge about barramundi spermatozoa and milt is limited. Barramundi spermatozoa were first described in Jamieson and Leung (1991), and two cryopreservation studies have been published using spermatozoa collected from wild-caught broodstock (Leung, 1987; Palmer et al., 1993). In both studies, the Marine Ringer's solution (MRS) was used as the base medium for sperm cryopreservation and artificial insemination trials (Leung, 1987; Palmer et al., 1993). Fresh, chilled, and frozen-thawed spermatozoa were used to fertilize ova successfully, indicating that the MRS used was sufficient to maintain barramundi sperm viability during short-term chilled storage (Leung, 1987; Palmer et al., 1993). However, in our laboratory, when spermatozoa collected from captive-bred barramundi broodstock were diluted in MRS according to Palmer et al. (1993), spermatozoa rapidly lost cell membrane integrity and lysed within 30 min (Appendix A). Therefore, further investigation of the biochemical composition of barramundi milt was warranted to optimize the NAM for use on spermatozoa derived from industry-standard captive-bred barramundi.

This present study investigated: (i) the biochemical properties of barramundi seminal and blood plasma to gain a greater understanding of the barramundi internal physiology; (ii) the influence of several components of the diluent, including osmolality, pH, and Na^+ and K^+ concentration on sperm quality; and (iii) the ability of the optimized NAM from (i) and (ii) to retain the viability and motility of barramundi spermatozoa after short-term chilled storage.

4.3 Methods

4.3.1 Animals

Captive-bred barramundi were maintained at the Marine and Aquaculture Research Facility (MARF), James Cook University, Townsville, Australia. Two cohorts of male fish were sampled: (i) eight male barramundi (mean body weight: 3.5 ± 0.2 kg, length: 62.0 ± 1.4 cm) that were maintained in 2,500 L tanks at 28 °C, 30 ppt salinity, and 16 h light: 8 h dark cycle for blood plasma sampling, and (ii) ten male broodstock (mean body weight: 3.9 ± 0.6 kg, length: 70.1 ± 4.8 cm) that were maintained in 28,000 L tanks under standard breeding conditions (i.e., 30 °C, 30 ppt salinity, and 16 h light: 8 h dark cycle) and were sampled for seminal plasma and spermatozoa. Experiments were approved by the James Cook University Animal Ethics Committee (A2406).

4.3.2 Sample collection and preparation

Blood was collected from the caudal vein of sedated fish using 18G 3.5" spinal needles (Provet Qld Pty Ltd, Brisbane, Australia). A total blood volume of 3 mL was extracted from each fish. Blood was centrifuged immediately after collection for 10 min at 10,000 rpm. Blood plasma was obtained by pipetting the supernatant into an Eppendorf tube and stored at -80 °C until use for analysis. Sperm samples were collected as described in Chapter 3; Marc et al. (2021). Briefly, prior to sperm collection, broodstock were sedated in an anaesthetic bath containing 40 mg/L iso-eugenol (AQUI-S®, New Zealand), and the gonopore was rinsed with distilled water. Milt samples were retrieved from the gonads by cannulation and an aliquot assessed for quality control (i.e., presence of blood, urine contamination, premature sperm activation, or failure to activate in contact with saltwater) before being deemed

suitable for inclusion in subsequent trials. Milt was centrifuged immediately after collection. Seminal plasma was pipetted into an Eppendorf tub after centrifuging the semen at 3000 g for 10 min and stored at -80 °C until used for analysis.

4.3.3 Biochemical composition and osmolality

Blood and seminal plasma were analyzed for 12 biochemical parameters (Sodium, Na^+; Potassium, K^+; Calcium, Ca^{2+}; Magnesium, Mg^{2+}; Chlorine, Cl^-; Phosphate, PO_4^{3-}; partial pressure of Carbon Dioxide, pCO_2; Total Protein, Cholesterol, Glucose, Triglyceride, and Urea) using an Olympus AU 480 Clinical Chemistry Analyzer (Beckman Coulter, USA). Osmolality was measured by a freezing point depression osmometer (Osmomat 030; Genotec GmbH, Berlin, Germany) and pH recorded using a micro-pH electrode (inlab ultra-micro-ISM; Mettler-Toledo, Melbourne, Australia).

4.3.4 Sperm function assessment

Sperm samples were assessed for motility and viability following methods described in Chapter 3; Marc et al. (2021). Sperm motility was activated by dilution with natural seawater consisting of 404.9 mM Na^+, 459.1 mM Cl^-, 10.1 mM K^+, 9.7 mM Ca^{2+}, 0.1 mM PO_4^{3-}, pH 8.1, and 900 mOsm/kg. Sperm motility parameters were evaluated by a computer-assisted sperm analyzer (CASA) system consisting of a light microscope (Olympus CX41; Olympus, Tokyo, Japan) equipped with a 20 X negative phase-contrast objective coupled with a 0.5 X C-Mount adaptor. The video was acquired at 60 fps by a digital camera (Basler avA1000-100gc, Basler AG, Ahrensburg, Germany) and pre-processed by CASA software (AndroVision®, version 1.1, Minitüb GmbH, Tiefenbach, Germany). The analysis of motility was performed in triplicate for each fish. Sperm viability was assessed using a dual staining method Hoechst 33342/Propidium Iodide (PI; nuclear stains) by flow cytometry (CyanADP flow cytometer, Beckman Coulter, Fullerton, CA, USA). Flow cytometry data were analyzed using Summit version 4.4 software (Beckman Coulter, Fullerton, CA, USA).

4.3.5 Experimental design

Marine Ringer's solution (MRS), described by Palmer et al. (1993), was used as a starting media for developing an optimized NAM. MRS consisted of 124.1 mM NaCl, 5.1 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $NaHCO_3$, 2.6 mM $NaH_2PO_4 \cdot 2H_2O$, and 5.6 mM D^+ glucose, adjusted to pH 7.4 with a final osmolality of 260 mOsm/kg.

4.3.5.1 Trial 1. Effect of $NaHCO_3$ buffered NAM osmolality on sperm quality

After 30 min incubation in MRS, preliminary observation of barramundi spermatozoa showed extensive head swelling and cell lysis, indicative of hypotonic shock (Appendix A). Therefore, this trial aimed to determine whether an increase in osmolality from 260 mOsm/kg to values that more closely mimic barramundi' seminal plasma (i.e., 340–450 mOsm/kg) could restore osmotic balance and improve barramundi sperm viability. The effect of osmolality on sperm viability was assessed by increasing osmolality with additional NaCl to create treatments of 300 mOsm/kg, 350 mOsm/kg, 400 mOsm/kg, and 450 mOsm/kg. Sperm samples from five males were initially diluted at 1:10 in different NAM and incubated at 4 ℃ for 1 h. Sperm motility and sperm viability were then assessed.

4.3.5.2 Trial 2. Effect of $NaHCO_3$ buffered NAM pH on sperm motility

Extracellular parameters, such as pH, are crucial for the retention of motility in fish spermatozoa (see review Alavi and Cosson, 2005). Therefore, after determining the optimal osmolality to prevent cell lysis, the effect of pH ranging from 6.5 to 8.5 was investigated to improve the activation potential of spermatozoa following chilled storage in barramundi. Specifically, the pH of the NAM was adjusted to pH 6.5 to determine if acidic conditions inhibited sperm motility, pH 7.4 to replicate the previously published formula (Palmer et al., 1993), pH 7.8 to mimic blood and seminal plasma pH, pH 8.1 to replicate external water pH used in aquaculture, and pH 8.5 to mimic the seawater pH level. For this experiment, the optimal NAM determined in trial 1 was selected, which consisted of 182.4 mM NaCl, 5.1 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $NaHCO_3$, 2.6 mM $NaH_2PO_4 \cdot 2H_2O$, and 5.6 mM D^+ glucose with a final osmolality of 400 mOsm/kg and pH was adjusted to the required

level using 0.1 M NaOH. Sperm samples from ten fish were undiluted (control) or diluted at 1:10 in the different extenders and incubated at 4 ℃ for 1 h and 24 h before motility was assessed by CASA.

4.3.5.3 Trial 3. Effect of HEPES buffered NAM pH on sperm motility

Trial 2 and other trials (Appendix B) indicated that the $NaHCO_3$ based buffering agents might inhibit the motility of barramundi spermatozoa. Therefore, the pH trial was repeated using HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, as it is known to have improved buffering stability at low temperatures compared to $NaHCO_3$ based buffers (Matthews et al., 2018; Williams-Smith et al., 1977). For this experiment, the NAM used consisting of 185 mM NaCl, 5.1 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 10 mM HEPES, and 5.6 mM D^+ glucose with a final osmolality of 400 mOsm/kg and pH adjusted to the required value using 0.1 M NaOH. Sperm samples from seven fish were undiluted (control) or diluted at 1:10 in the different media and incubated at 4 ℃ for 1 h and 24 h before motility was assessed by CASA.

4.3.5.4 Trial 4. Effect of HEPES buffered NAM sodium and potassium concentration on sperm motility

Extracellular Na^+ and K^+ concentrations are known to play a major role in maintaining activation potential in spermatozoa (see review Alavi and Cosson, 2006). Therefore, the effect of Na^+ and K^+ concentration ratios on sperm motility was tested following chilled storage. The optimal NAM determined in Trial 3 was selected, and different concentration ratios of Na^+ and K^+ were tested. Different HEPES buffered NAM were prepared with the following NaCl/KCl concentrations: 0 mM NaCl/190 mM KCl, 140 mM NaCl/50 mM KCl, 160 mM NaCl/30 mM KCl, 185 mM NaCl/5 mM KCl, and 190 mM NaCl/0 mM KCl. All solutions maintained an osmolality of 400 mOsm/kg and were adjusted to pH 7.4 with 1 M KOH for 0 mM NaCl/190 mM KCl treatment group or 0.1 M NaOH for all other treatment groups. The first and last treatment groups, 0 mM NaCl/190 mM KCl and 190 mM NaCl/0 mM KCl, respectively, were designed to determine whether the motility of barramundi spermatozoa is Na^+ or K^+ dependent, while the intermediate treatments spanned the breadth of seminal

plasma ionic measurements. Sperm samples from six males were undiluted (control) or diluted at 1:10 in the different NAM and incubated at 4 ℃ for 1 h and 24 h before motility was assessed by CASA.

4.3.5.5 Trial 5. Evaluation of the optimized NAM for short-term chilled storage of barramundi milt

To quantify the performance of the optimized NAM, the motility of spermatozoa diluted in the NAM (derived from Trial 4) was compared to that of spermatozoa stored undiluted (control) after 1 h, 24 h, 48 h, 72 h, and 96 h incubation at 4 ℃.

4.3.6 Statistical analysis

Statistical analyses were performed using RStudio version 1.0.153 (RStudio, Inc, Boston, MA, USA). Percentage data for motility (total motility, TM; progressive motility, PM; slow, medium, fast), kinetic parameters (linearity, LIN; straightness, STR; wobble, WOB), and viability were normalized using arcsine ($\sqrt{value/100}$) transformation. All data were then assessed for normality and homogeneity of variance using Shapiro-Wilk and Levene tests. Where data were normally distributed with homogeneous variance, one-way ANOVA, followed by Tukey's multiple comparison test, was used to test differences between treatments. A Kruskal-Wallis H test, followed by Fisher's Least Significant Difference test was performed when these assumptions were not met to compare treatment pairs. The paired samples Wilcoxon test (Wilcoxon signed-rank test) was used to evaluate significant differences between 1 h and 24 h incubation for Trials 2, 3, and 4. Kruskal-Wallis rank-sum test followed by pairwise Mann-Whitney U-tests with Bonferroni correction was used to assess the significance between groups at each time point for Trial 5. Data are displayed as mean ± standard error (SEM). The level of significance was set at $P < 0.05$.

4.4 Results

4.4.1 Biochemical analysis

Ionic and metabolite concentrations of blood plasma were relatively homogenous between individual barramundi. This was in contrast with that observed for seminal plasma, where substantial individual variation occurred (Table 4.1). Na^+ and Cl^- concentrations of blood and seminal plasma were found not significantly different, as were the concentrations of organic components pCO2, glucose, triglyceride, and urea (Table 4.1). However, the concentration of K^+ and PO_4^{3-} were significantly higher in seminal plasma than blood plasma (i.e., 10-fold; Welch t-test, K^+: $t(7.02) = -8.74$, $P < 0.001$, PO_4^{3-}: $t(7.02) = -6.26$, $P < 0.001$; Table 4.1). Mg^{2+} concentration was also significantly higher in seminal plasma than blood plasma (Wilcoxon's test, $P < 0.001$, r = -0.89; Table 4.1). The higher concentration of these ions in seminal plasma is consistent with an overall 18.8% increase in osmolality (mean: 396.1 ± 13.4 mOsm/kg) over blood plasma (mean: 333.5 ± 2.1 mOsm/kg; Table 4.1). By contrast, Ca^{2+}, total protein, cholesterol, and pH were significantly lower in seminal plasma than blood plasma (Table 4.1).

Table 4.1 Mineral and organic composition of seminal and blood plasma of captive barramundi (*Lates calcarifer*) broodstock reared in saltwater.

	Blood Plasma			Seminal Plasma		
Parameter	**Mean ± SEM (*n*)**	**Range**	**CV**	**Mean ± SEM (*n*)**	**Range**	**CV**
Na^+ (mM)	160.7 ± 0.9[a] (8)	155.8 - 163.6	1.7	164.2 ± 5.1[a] (8)	140.7 - 181.8	9.4
K^+ (mM)	3.7 ± 0.1[a] (8)	3.1 - 4.3	11.8	37.7 ± 3.6[b] (8)	23.3 - 53.3	29.2
Ca^{2+} (mM)	2.6 ± 0.1[a] (8)	2.4 - 3.0	6.9	1.5 ± 0.3[b] (8)	0.5 - 2.9	59.4
Mg^{2+} (mM)	1.0 ± 0.0[a] (8)	0.9 - 1.1	5.4	3.9 ± 1.1[b] (8)	1.7 - 12.2	86.3
Cl^- (mM)	133.4 ± 1.0[a] (8)	129.4 - 137.8	2.2	149.7 ± 7.2[a] (8)	129.0 - 198.8	14.5
PO_4 (mM)	1.5 ± 0.1[a] (8)	1.1 - 1.8	16.4	15.4 ± 2.1[b] (8)	7.9 - 27.3	40.7
pCO_2 (mmHg)	2.6 ± 0.1[a] (8)	2.4 - 3.0	6.3	5.6 ± 0.8[a] (8)	2.0 - 10.0	44.5
TP ($g.L^{-1}$)	44.3 ± 1.4[a] (8)	36.4 - 49.6	9.7	28.5 ± 4.1[b] (6)	14.4 - 46.0	38.8
CHO (mM)	5.6 ± 0.5[a] (8)	4.2 - 8.2	25.8	1.7 ± 0.5[b] (7)	0.0 - 3.0	83.0
Glu (mM)	3.7 ± 0.4[a] (8)	2.3 - 5.9	33.0	2.1 ± 0.4[a] (8)	0.4 - 3.9	60.7
TG (mM)	1.0 ± 0.2[a] (8)	0.5 - 2.3	58.3	3.7 ± 1.9[a] (4)	1.1 - 10.2	117.1
Urea (mM)	2.4 ± 0.1[a] (8)	2.1 - 2.9	10.8	4.2 ± 0.8[a] (3)	2.3 - 5.3	39.7
pH	7.8 ± 0.0[a] (8)	7.7 - 7.9	0.9	7.5 ± 0.1[b] (5)	7.2 - 7.8	3.6
Osm (mOsm/kg)	333.5 ± 2.1[a] (8)	325 - 341	1.9	396.1 ± 13.4[b] (7)	340 - 451	9.7

Data are displayed as mean ± standard error (SEM), number of fish (*n*), and coefficient of variation (CV) are reported for each characteristic. Different letters indicate significant differences ($P < 0.05$) between blood and semen. Abbreviations: Total protein (TP), cholesterol (CHO), glucose (Glu), triglycerides (TG), and osmolality (Osm).

4.4.2 Trial 1. Effect of $NaHCO_3$ buffered NAM osmolality on sperm quality

The highest viability of barramundi spermatozoa was observed when stored in NAMs that mimicked the range of seminal plasma osmolality (350–450 mOsm/kg; Table 4.1; Figure 4.1). In particular, the viability of barramundi spermatozoa was significantly higher after 1 h incubation in NAM adjusted to 400 mOsm/kg (78.2 ± 2.9%) when compared to the original NAM at 260 mOsm/kg (44.8 ± 3.3%; ANOVA, $F(1, 23) = 11.18$, $P < 0.05$; Figure 4.1). However, sperm motility did not differ significantly between NAMs and remained low (23.5 ± 2.1%), even with the increase in viability of spermatozoa held in NAM adjusted to 400 mOsm/kg.

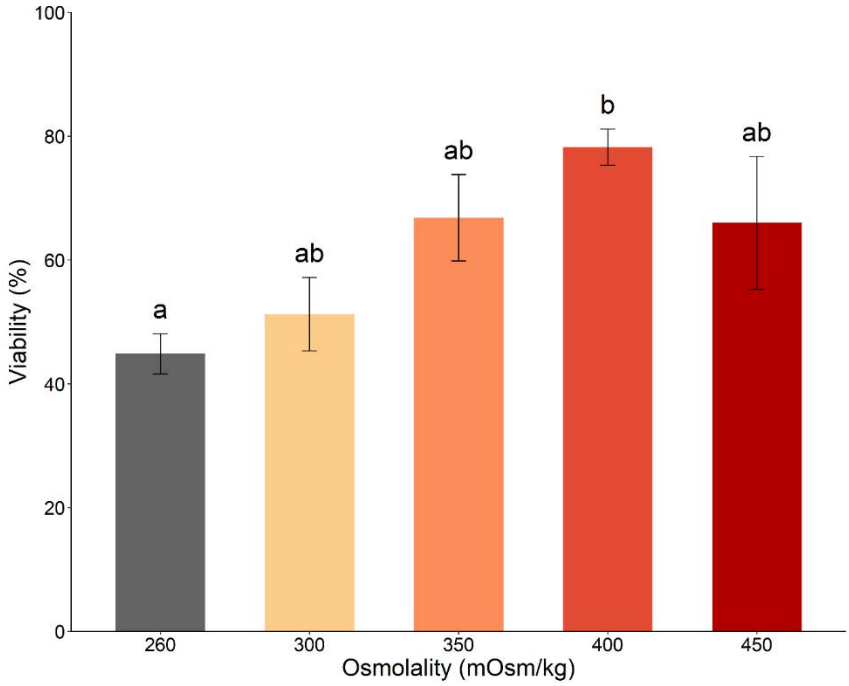

Figure 4.1 Percentage of live barramundi (*Lates calcarifer*) spermatozoa ($n = 5$) after 1 h incubation at 4 °C in $NaHCO_3$ buffered non-activating medium with an osmolality of 260 to 450 mOsm/kg. Data are displayed as mean ± SEM. Different lowercase letters indicate a significant difference between treatments ($P < 0.05$).

4.4.3 Trial 2. Effect of $NaHCO_3$ buffered NAM pH on sperm motility

After 1 h incubation, spermatozoa held in NAM with a pH between 7.4 and 8.5 (mimicking the range between seminal plasma and natural seawater) showed an unexpected and significant decline in total motility compared to the control (ANOVA, $F(5, 54) = 9.81$, $P < 0.01$; Figure 4.2; Appendix C). Interestingly, there were no significant differences in sperm swimming velocities (VCL, VSL, and VAP) between NAMs (Appendix C). After 24 h incubation, sperm samples were functionally immotile in media between pH 7.4 to 8.5, but remained motile in the control and pH 6.5 medium, although total motility declined significantly for both media compared to the 1 h incubation (Wilcoxon's test, Control: $P = 0.002$, r = 0.89; pH 6.5: $P = 0.0039$, r = 0.854; Figure 4.2; Appendix C). The motility of spermatozoa stored at pH 6.5 did not differ from control milt at either incubation period (Figure 4.2; Appendix C). Of note, chemical analysis of NAMs showed an increase of pCO2 in relation to the increase of pH (pH 6.5: 0.0 mmHg; pH 7.4: 1.3 mmHg; pH 7.8: 2.4 mmHg; pH 8.1: 3.2 mmHg; pH 8.5: 4.5 mmHg).

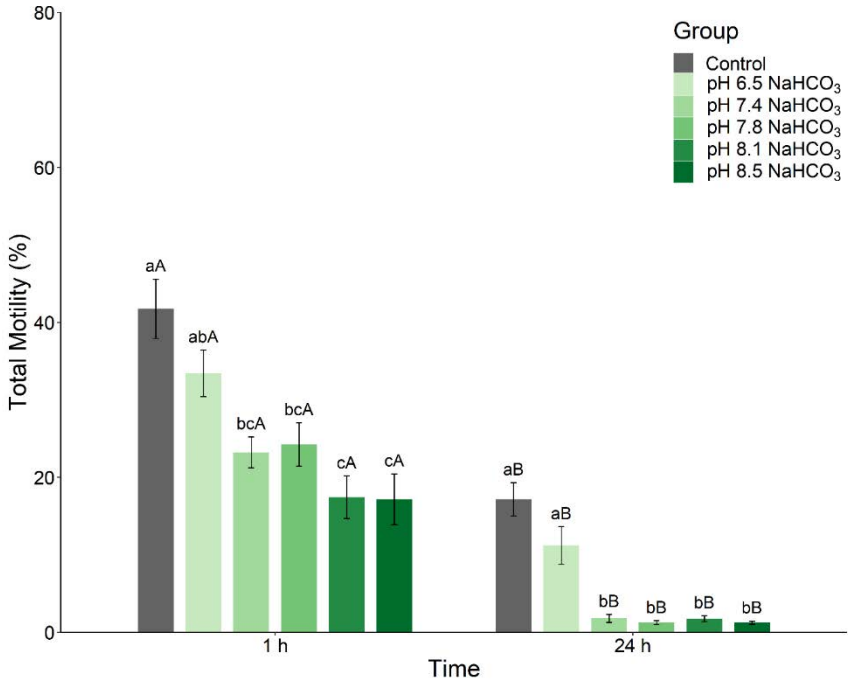

Figure 4.2 Total motility of barramundi (*Lates calcarifer*) spermatozoa ($n = 10$) incubated for 1 h and 24 h at 4 °C undiluted (control) or diluted in $NaHCO_3$ buffered non-activating medium with different pH. Data are displayed as mean ± SEM. Different lowercase letters indicate a significant difference between pH treatments within an incubation period, and different capital letters indicate a significant difference between incubation periods at the same pH ($P < 0.05$).

4.4.4 Trial 3. Effect of HEPES buffered NAM pH on sperm motility

After 1 h incubation, sperm motility and kinetic parameters remained high across all NAMs, with no significant differences between media (Figure 4.3; Appendix D). Spermatozoa held in the pH 7.8 medium showed the highest total motility at 55.8 ± 4.0%, followed by pH 7.4 (51.8 ± 7.5%). After 24 h incubation, total motility declined significantly for all groups, including the control, compared to when assessed at 1 h (Wilcoxon's test, P = 0.031, r = 0.898; Figure 4.3; Appendix D). The pH 7.4 and 7.8 media and the control group retained the highest total motility with 35.1 ± 3.3%, 29.7 ± 3.4%, and 28.1 ± 6.3% respectively, and were significantly different for this parameter when compared to other groups after 24 h incubation (ANOVA, $F(6, 35) = 12.23$, $P < 0.001$; Figure 4.3; Appendix D). Spermatozoa held in pH 6.5 ($NaHCO_3$ and HEPES buffered), 8.1, and 8.5 media showed a significant decline in sperm motility (TM, PM, and Fast) and kinetic (VCL, VSL, VAP, and ALH) compared to the pH 7.4 and 7.8 media (Appendix D). Of note, except for BCF, there was no significant difference in sperm motility characteristics between $NaHCO_3$ or HEPES buffered NAM at pH 6.5 after incubation for 1 h or 24 h (Figure 4.3; Appendix D).

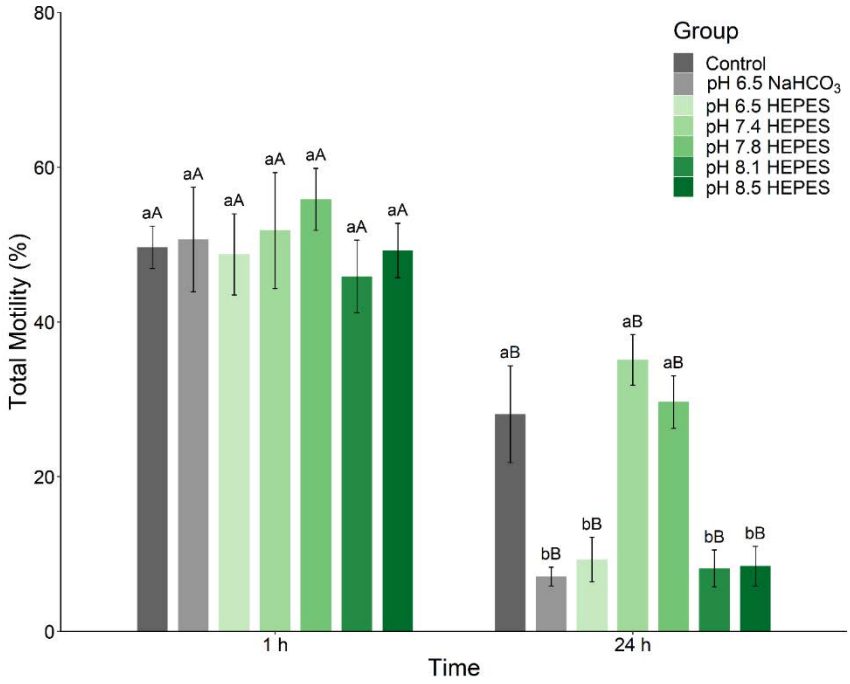

Figure 4.3 Total motility of barramundi (*Lates calcarifer*) spermatozoa (n = 7) incubated for 1 h and 24 h at 4 °C undiluted (control) or diluted in HEPES buffered non-activating medium with different pH. Data are displayed as mean ± SEM. Different lowercase letters indicate a significant difference between pH treatments within an incubation period, and different capital letters indicate a significant difference between incubation periods at the same pH ($P < 0.05$).

4.4.5 Trial 4. Effect of HEPES buffered NAM Na^+ and K^+ concentrations on sperm motility

After 1 h, spermatozoa incubated in Na^+ free medium showed a large decline in sperm motility (4.6 ± 0.6%) compared to the undiluted milt control (43.5 ± 4.5%; ANOVA, $F(5, 30) = 20.88$, $P < 0.001$; Figure 4.4; Appendix E). By contrast, the K^+ free (0 mM) and low K^+ (5 mM) media with the highest Na^+ concentrations had the highest total motility (56.5 ± 4.3% and 56.8 ± 5.0% respectively; Figure 4.4; Appendix E). A similar trend was observed for progressive motility, fast motility, curvilinear, straight line, and average path velocities (VCL, VSL, and VAP; Appendix E). In particular, the 185/5 mM NaCl/KCl medium showed the highest progressive motility (28.5 ± 2.4%) with most spermatozoa classified as fast (26.4 ± 2.3%), and a high overall sperm velocity (VCL: 112.1 ± 7.4 μm/s, VSL: 79.1 ± 6.8 μm/s, and VAP: 102.6 ± 7.6 μm/s). For all media containing Na^+, the swimming trajectory of spermatozoa (LIN, STR, WOB, ALH, and BCF) showed limited deviation from the primary path (Appendix E). After 24 h incubation, the highest total motility was also recorded in the NAM containing 185 mM NaCl/5 mM KCl (47.7 ± 7.2%), which did not differ from that initially observed after 1 h, indicating the ability of this NAM to maintain sperm activation potential during temporary chilled storage (Wilcoxon's test, $P = 0.313$, r = 0.469; Figure 4.4; Appendix E). Of note, this trial was initially conducted using the $NaHCO_3$ buffered NAM at pH 7.4, 400 mOsm/kg (Appendix B). However, the influence of NaCl and KCl concentration on sperm motility was masked due to the presence of $NaHCO_3$ buffers (Figure 4.2 and Figure 4.3; Appendix B).

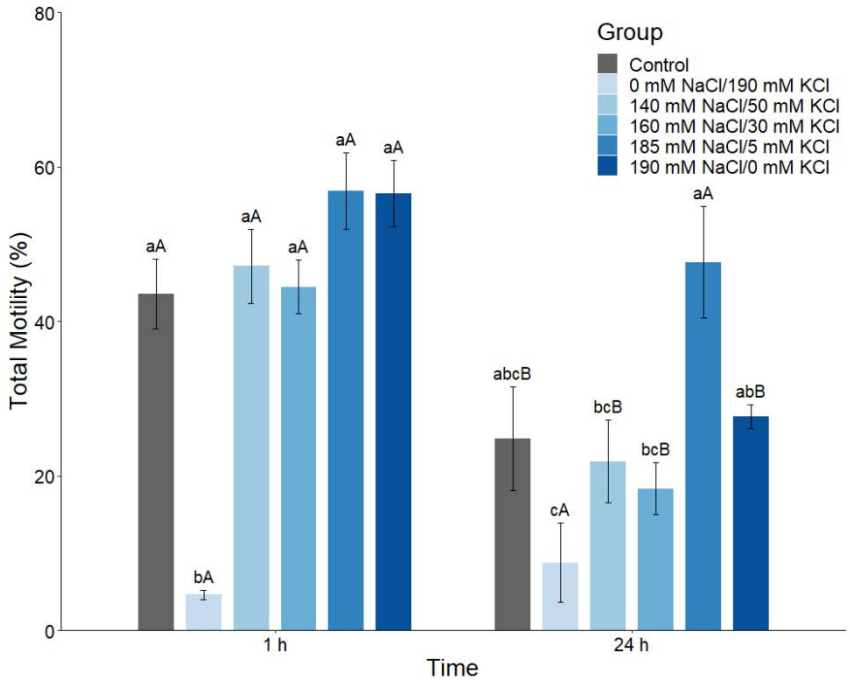

Figure 4.4 Total motility of barramundi (*Lates calcarifer*) spermatozoa (n = 6) incubated for 1 h and 24 h at 4 °C undiluted (control) or diluted in HEPES buffered non-activating medium containing different concentrations of NaCl and KCl. Data are displayed as mean ± SEM. Different lowercase letters indicate a significant difference between concentration treatments within an incubation period, and different capital letters indicate a significant difference between incubation periods at the same concentration treatment ($P < 0.05$).

4.4.6 Trial 5. Evaluation of the optimized NAM for short-term chilled storage of barramundi milt

After 1 h incubation, spermatozoa held in the NAM showed significantly higher motility and trajectory (TM, PM, Fast, VCL, VSL, VAP, LIN, and WOB) than those in the undiluted milt control (Figure 4.5; Appendix F). Other swimming trajectories (STR, ALH, and BCF) did not differ between both groups. Total sperm motility only declined significantly after 72 h in the optimized NAM (Kruskal-Wallis Rank Sum test; H = 16.68, df = 4, $P < 0.002$) compared to 48 h for the undiluted milt control (Kruskal-Wallis Rank Sum test; H = 18.45, df = 4, $P = 0.001$; Figure 4.5; Appendix F). Of note, for both of these time points, the decrease in average motility and velocity was mainly due to the non-activation of sperm motility in samples from two fish at 48 h and three fish at 72 h, rather than a ubiquitous drop in motility and kinetic values across fish (Appendix G). With excluding samples that failed activation, total motility was 42.6 ± 6.0% at 48 h and 31.6 ± 2.2% at 72 h, respectively (Appendix G). After incubation

of spermatozoa for 96 h, total motility was less than 5%, and there was an absence of progressive motility, with all diluted and undiluted samples (Figure 4.5; Appendix F).

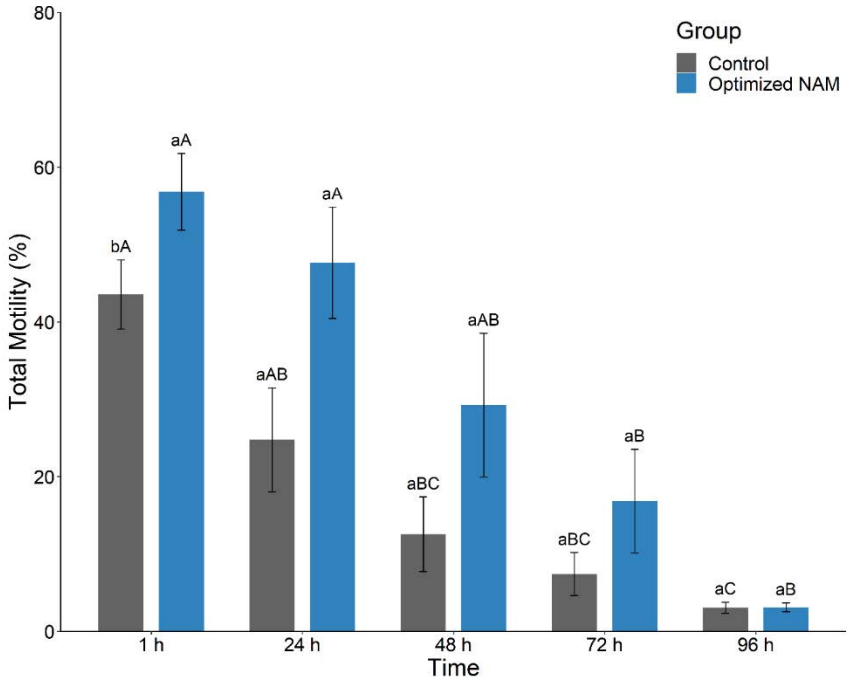

Figure 4.5 Motility of barramundi (*Lates calcarifer*) spermatozoa (n = 6) incubated for up to 96 h at 4 °C undiluted (control) or diluted in optimized HEPES buffered non-activating medium. Data are displayed as mean ± SEM. Different lowercase letters indicate a significant difference between extender treatments within an incubation period, and different capital letters indicate a significant difference between incubation periods for the same medium ($P < 0.05$).

4.5 Discussion

Reliable sperm handling and storage protocols are required to establish artificial breeding techniques (e.g., artificial fertilization, milt banking) for the barramundi aquaculture industry. However, despite previously published protocols for the handling and storing spermatozoa from wild-caught barramundi (Leung, 1987; Palmer et al., 1993), such methods were unsuccessful for use with commercial captive-reared broodstock spermatozoa. In this study, we found that the presence of $NaHCO_3$ and an osmotic imbalance in the previously published medium inhibited sperm motility and caused lysis of the barramundi sperm plasma membrane. Furthermore, the characterization of ionic and metabolite compositions of barramundi blood and seminal plasma provided the fundamental knowledge required to develop an optimized NAM. The optimized NAM allowed chilled storage of testicular spermatozoa

collected from captive-bred barramundi for 48 h with no significant loss of total motility. Furthermore, while partially reduced, the activation potential of diluted spermatozoa was retained for up to 72 h and remained consistently higher than undiluted samples until the complete loss of motility was observed at 96 h.

Characterization of the ionic composition and osmolality of blood and seminal plasma provides valuable insight into the potential cause of damage to spermatozoa stored in previously published MRS (Palmer et al., 1993). Of particular note, the original MRS contained 124.1 mM of Na^+ and had a total osmolality of 260 mOsm/kg, which was much lower than seminal (mean: 396.1 ± 13.4 mOsm/kg) and blood plasma (mean: 333.5 ± 2.1 mOsm/kg) derived from captive-reared barramundi held under commercial conditions (i.e., 30 ppt). Moreover, the Na^+ concentration and osmolality of MRS were similar to blood plasma of captive-reared barramundi held in freshwater (Na^+: 105.6 ± 2.8 mM and 218.3 ± 3.3 mOsm/kg; unpublished data). This suggests MRS may be isotonically balanced with spermatozoa collected from barramundi from freshwater, or low-salinity/brackish environments, rather than marine salinities (i.e., 30–35 ppt) in which they spawn. Milt collected by Palmer et al. (1993) were from wild barramundi captured in an estuary at the start of the monsoon season (i.e., November) in north Queensland, Australia, where there may have been freshwater plumes. The different environmental conditions may be a potential source of discrepancy, altering milt osmolality and thus the suitability of MRS observed within our study. In Atlantic cod (*Gadus morhua*), the ionic and metabolite composition of seminal plasma can differ significantly across the spawning season and between wild-caught and captive-bred fish (Anthony et al., 2011; Butts et al., 2010). Furthermore, Northern pike (*Esox Lucius*) ionic concentrations and osmolality differ in seminal plasma collected from the gonad either by cannulation (mean: 358 ± 77 mOsm/kg) or by manual striping (mean: 273 ± 21 mOsm/kg; Hulak et al., 2008). As such, differences in the origin of fish (i.e., wild *vs.* captive-bred) and collection technique (i.e., testicular cannulation in our study *vs.* manual stripping) with Palmer et al. (1993) may explain the poor performance of MRS in our study. Thus, future research is warranted to characterize the ionic composition of milt derived from manually stripped captive barramundi.

In this study, the presence of $NaHCO_3$ in the NAM did not affect sperm motility at pH 6.5 but caused it to decline significantly at pH 7.4 or greater. The only discernible difference between the media at pH 6.5 and those ≥ 7.4 was the increase of pCO_2/HCO_3^- concentration, in line with the increase in pH of the medium. $NaHCO_3$ dissociates in aqueous solution into $CO_2 + H_2CO_3$ (free CO_2), HCO_3^-, and CO_3^{2-} with the equilibrium ratio of each chemical species present being pH-dependent (Burt and Rau, 1994). At pH 7.4 and above, free-CO_2 transmutes into HCO_3^- and H^+, whereas at pH 6.5, free-CO_2 does not fully dissociate and remains a gas at equilibrium. These results suggest three possible scenarios. Firstly, barramundi spermatozoa may take up HCO_3^- through osmosis using Cl^-/HCO_3^- and Na^+/HCO_3^- exchanger proteins, such as those used by mammalian spermatozoa to transport HCO_3^- directly into the cytoplasm (see review Nishigaki et al., 2014). An increase in internal HCO_3^- concentration causes the intracellular accumulation of H^+ through the K^+ channel, leading to a drop in internal pH that limits further H^+ uptake necessary for the initiation of motility when spermatozoa are exposed to saltwater (Inaba and Dreanno, 2003; Tanaka et al., 2002). Secondly, in Japanese eel, CO_2 was the inhibitory factor of $NaHCO_3$ because the internal increase in HCO_3^- and H^+ was mediated by conversion of CO_2 by carbonic anhydrase, limiting further H^+ uptake required for the initiation of motility (Tanaka et al., 2002). Similarly, in turbot (*Scophthalmus maximus*), sperm motility was inhibited by HCO_3^- through its direct action on the flagellum axoneme, in which an intracellular increase in HCO_3^- concentration was caused by CO_2 conversion (Inaba and Dreanno, 2003). Lastly, elevated CO_3^{2-} concentration in solutions with high pH can cause precipitation of Ca^{2+} in the form of $CaCO_3$. In our study, a reduction in bioavailable Ca^{2+} in the medium may have inhibited sperm motility at these higher pH values. Biochemical analysis of our NAMs did not show a difference in Ca^{2+} concentration at different pH (unpublished data). However, some precipitation during chilled storage was observed in-stock solutions that contained $NaHCO_3$ buffers. Thus, the concentration of bioavailable Ca^{2+} may have differed between NAMs of different pH during sperm storage, which may have contributed to the lower motility at pH greater than 7.4 seen in our study. Further studies are required to elucidate the detailed molecular and ionic mechanism regulating sperm motility in barramundi.

Critically, when $NaHCO_3$ was replaced with the HEPES buffer, the activation potential of spermatozoa was retained across the complete pH range after 1 h of incubation and was highest after 24 h when incubated in NAMs with pH most similar to seminal plasma (i.e., pH 7.4 and 7.8). In goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*), a significant decrease in sperm motility and viability was observed when spermatozoa were stored in acidic conditions (Billard et al., 1995; Chantzaropoulos et al., 2015). In our study, barramundi sperm motility declined after 24 h in both acid (pH 6.5) and alkaline ($pH \leq 8.1$) environments. It is well accepted that mitochondrial ATP-synthase can only function optimally within a narrow pH range that is species-specific (Alavi and Cosson, 2005; Vílchez et al., 2017), suggesting that ATP-synthase may not generate sufficient ATP to drive flagella function outside the pH 7.4–7.8 range for barramundi spermatozoa. At the level of individual fish, the variation in optimal pH, ranging between 7.4 and 7.8, could indicate a different degree of sperm maturation. Changes in seminal plasma pH from 7.5 to 8.0 have been linked to the progressive maturation of spermatozoa in rainbow trout (*Oncorhynchus mykiss*; see review Alavi and Cosson, 2005) and in Japanese eel (Billard et al., 1995; Ohta et al., 1997). Further studies should assess if the maturation stage of barramundi affects seminal plasma pH, along with whether smaller increments of pH between 7.4 and 7.8 provide improved motility.

While the motility achieved in the optimized NAM was substantially improved throughout this study from 20% initially to ~60%, the total motility is lower than reported in many species (e.g., 90% in common carp; Cejko et al., 2018, and in *Prochilodus lineatus*; Viveiros et al., 2016, ~80% in red seabream *Pagrus major*; Liu et al., 2007) and is less than otherwise would be expected for barramundi (Leung, 1987; Palmer et al., 1993). Yet, the low motility generally found in our study is concomitant with levels found in spermatozoa derived from testicular cannulation compared to manual stripping in both turbot (Suquet et al., 2000) and Japanese eel (Ohta et al., 1997). Moreover, the motility of testicular spermatozoa in Japanese eel could be acquired artificially in a medium containing a high concentration of K^+ and HCO_3^- (Ohta et al., 1997). This phenomenon was not observed in our study, in which the presence of HCO_3^- inhibited sperm motility, irrespective of Na^+ and K^+ concentration. Furthermore, similar to the European eel (Vílchez et al., 2016), barramundi spermatozoa appear to require Na^+ in the

NAM to maintain activation potential as sperm motility was rapidly inhibited within 1 h in Na^+ free medium. However, in contrast to the Japanese eel (Ohta et al., 2001) and the European eel (Vílchez et al., 2017, 2016), barramundi spermatozoa exhibit improved motility at low K^+ concentration (i.e., 5 mM), similar to that in blood plasma (3.7 ± 0.1 mM) rather than high K^+ concentration (i.e., 30 mM) as found in seminal plasma (37.7 ± 3.6 mM). Further research is needed to characterize barramundi sperm physiology and ionic mechanisms such as determining whether smaller increments of K^+ between 0 and 30 mM could improve motility.

Lastly, we have demonstrated that spermatozoa collected by testicular cannulation from commercial captive-reared barramundi can be stored in the optimized NAM at 4 ℃ for up to 48 h without any significant loss of total motility. Moreover, spermatozoa can retain their activation potential after up to 72 h chilled storage in this medium. This offers the possibility to hold barramundi milt temporally for 2 to 3 days which, once a reliable stripping technique to obtain oocytes become available, will facilitate the development of tightly controlled artificial fertilization programs for barramundi.

4.6 Conclusion

This study provides an optimized NAM for the short-term chilled storage of barramundi spermatozoa collected by testicular cannulation. We demonstrated that the adjustment of the NAM's osmolality to 400 mOsm/kg and pH 7.4, similar to that of seminal plasma, was optimal for maintaining the viability and motility of barramundi spermatozoa. Furthermore, the replacement of $NaHCO_3$ with HEPES buffer was able to overcome $NaHCO_3^-$ induced inhibition of sperm motility. Finally, the inclusion of Na^+ at 185 mM and K^+ at 5 mM in the medium retained the best activation potential in barramundi spermatozoa. These results lay the foundation for further studies looking at the regulatory role of different ions in barramundi sperm motility to improve medium composition. More importantly, this optimized NAM facilitates safe handling and short-term storage of testicular spermatozoa, thereby facilitating the further development of advanced reproductive technologies for the species.

Chapter 5. Characterization of sperm quality in captive-bred barramundi (*Lates calcarifer*): effect on spawning performance and paternal contribution in mass-spawning events

5.1 Abstract

Barramundi (*Lates calcarifer*) is a species with a mass-spawning reproductive strategy, whereby adults synchronously release their gametes into the water column. In male barramundi, this spawning strategy usually results in differential broodstock contributions to progeny cohorts, creating skews in family size that hinder the effectiveness of selective breeding programs. As sperm quality is known to influence fertilization success and early larval development, the relationship between sperm quality of captive-bred broodstock and paternal contribution following mass-spawning events was investigated. Accordingly, we assessed the quality of sperm collected through cannulating male barramundi from three different breeding cohorts. Broodstock were allowed to spawn, and then offspring at 2.5 h and 12 h post-fertilization (hpf) and 24 h and 48 h post-hatching (hph) were collected. Offspring were assessed for morphological abnormalities, and key morphological parameters were recorded. Offspring collected at 2.5 hpf and 24 hph were also genotyped to determine their parentage and examine the relationship between sperm quality of individual males and offspring survival. Results highlighted several trends. Male physical condition and sperm quality were highly variable within each breeding cohort. Males with a lower physical condition showed lower sperm motility-velocity, whereas males with a higher physical condition showed higher sperm motility-velocity and higher levels of sperm DNA damage. In turn, while all males demonstrated fertilization capabilities, the paternal contribution was dominated by males with a lower physical condition. Moreover, a strong relationship between sperm DNA damage and larval survival ($r(22) = -0.67$, $P < 0.001$) was found, which could explain the reduced contribution of males with a higher physical condition to the progeny cohort. While this relationship was significant for the first spawning night, males with higher levels of sperm DNA damage were able to produce offspring surviving the early developmental stages in the second spawning night.

Consequently, sperm DNA damage might be associated with the presence of mature spermatozoa stored for an extended time in the spermatic duct rather than to the male fertility. Moreover, unmeasured variables, such as spawning behavior, social hierarchy, egg quality, and the spawning induction procedure might contribute to paternal skew in mass-spawning events, requiring further investigation. Finally, due to the complex dynamics occurring during mass-spawning events, further development of artificial reproductive technology is recommended to gain greater control over individual contribution and overcome current breeding bottlenecks.

5.2 Introduction

Barramundi (*Lates calcarifer*) has a reproductive strategy whereby adult male and female fish synchronously release their gametes in a coordinated mass-spawning event. In aquaculture, spawning events are generally conducted in a fashion that accommodates this mass-spawning behavior through hatcheries placing broodstock together in a large tank in a sex ratio of 1 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 broodstock contribution. As reported in many other 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 broodstock show a highly skewed paternal contribution to cohorts of progeny produced during a mass-spawning event (Domingos et al., 2014; Frost et al., 2006; Liu et al., 2012; Loughnan et al., 2013). Some studies have reported that despite having several males in the spawning group, one or two captive-bred barramundi broodstock often dominated paternal contribution and sired up to 55% of progeny, while other males contributed as little as 1% (Frost et al., 2006). Furthermore, this skewed paternal contribution seems to be accentuated in captive-bred males, as in comparison, mass-spawning events involving wild-caught barramundi broodstock showed a more even paternal contribution (Wang et al., 2008). This skewed contribution reduces the amount of genetic diversity captured and passed onto 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 the skewed paternal contribution of captive-bred individuals to overcome these bottlenecks.

To date, the causes of skewed paternal contribution 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). One potential cause of the differential paternal contribution observed in captive-male barramundi could be linked to variation in sperm quality of males within the spawning group. Specifically, diagnostics of sperm quality in several fish species

of commercial importance have highlighted natural inter-male variability of 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 Herráez et al., 2017). In particular, it has been demonstrated that DNA damage in spermatozoa 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; Zilli et al., 2003).

In commercial barramundi hatcheries, the current measures of sperm quality employed include a simple visual assessment of the sperm motility and milt color while also ensuring the fish have not undergone male to female sex change between spawning events, as barramundi is a protandrous hermaphroditic species (Jerry, 2013). Furthermore, some hatcheries forgo the assessment of sperm quality altogether. This absence or limited evaluation of the male reproductive condition is also applied when selecting new males to replace those which have sex changed, potentially allowing naturally subfertile 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 for barramundi (Chapter 3; Marc et al., 2021), it is now possible to reliably quantify broodstock sperm quality and investigate the association between broodstock sperm quality and the differential levels of paternal contribution observed in barramundi.

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 barramundi broodstock; (ii) to assess the relationship between sperm quality and paternal contribution to progeny cohorts resulting from mass-spawns; and (iii) to determine the relationship between sperm quality and early larval development in barramundi.

5.3 Methods

5.3.1 Animals

Captive-bred barramundi were maintained at the Marine and Aquaculture Research Facility (MARF), James Cook University, Townsville, Australia. Male broodstock were between 2 to 4 years old, and females were between 5 to 8 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; 16 h light: 8 h dark cycle. Tank A and B volumes were 28,000 L and contained 8 males and 4 females each, whereas Tank C volume was 18,000 L and contained 6 males and 3 females (Table 5.1). Fish were fed 4 days a week at 1% body weight with a formulated diet (LANSY-Breed Essential, INVE Aquaculture). Broodstock were identified by their unique Passive Integrated Transponder (PIT; Hallprint Fish Tags, AUS) tags. Experiments were approved by the James Cook University Animal Ethics Committee (A2406).

Table 5.1 Description of broodstock cohorts held in Tanks A, B, and C.

Tank	Number of fish/tank	Number of females/males	Male weight (kg)	Male length (cm)	Female weight (kg)	Female length (cm)	Stocking density (kg/m^3)
A	12	4/8	4.0 ± 0.3	70.7 ± 0.6	9.0 ± 0.4	86.1 ± 1.0	2.4
B	12	4/8	2.9 ± 0.3	67.2 ± 0.4	7.3 ± 0.3	86.0 ± 0.4	1.9
C	9	3/6	3.5 ± 0.2	73.1 ± 0.4	8.3 ± 0.2	88.5 ± 0.5	2.6

Data are displayed as mean ± standard error (SEM).

5.3.2 Sperm collection and assessment

Male broodstock were sedated in a saltwater bath containing 40 ppt AQUI-S® (Aquatic Diagnostic Services International) 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 cannula length to provide a relative measure of milt volume (Chapter 3; Marc et al., 2021). Sperm samples were assessed for motility directly after collection in the hatchery. The remaining sperm samples were then held on ice for about 3 h before being assessed for viability and DNA damage

in the laboratory using flow cytometry. Assessments were conducted according to Chapter 3; Marc et al. (2021). In short, 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 by a computer-assisted sperm analyzer (CASA) system consisting of a light microscope (Olympus CX41; Olympus, Tokyo, Japan) equipped with a 20 X negative phase-contrast objective. The video was acquired by a digital camera (Basler avA1000-100gc, Basler AG, Ahrensburg, Germany) at 60 fps interfaced with the CASA software (AndroVision, Minitüb GmbH, Tiefenbach, Germany). Viability and DNA integrity of spermatozoa were assessed using a dual-staining Hoechst 333242/Propidium Iodide (PI) method and transferase (TdT)-mediated dUTP nick-end labeling TUNEL (FITC/PI) assay, respectively. Dual-fluorescence of spermatozoa was measured by flow cytometry (FACS Canto™ II, BD Biosciences, San Jose, CA, USA) and data were analyzed using FlowJo software (Treestar, Inc., San Carlos, USA).

5.3.3 Spawning induction and egg collection

Spawning events were induced in all sedated males and females with vitellogenic oocytes by an intramuscular injection of luteinizing hormone-releasing analog (LHRHa; Aquatic Diagnostic Services International Pty., Ltd.; Thépot and Jerry, 2015). Each LHRHa injection triggered two consecutive nights of spawning, occurring on the second and third night after the 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; 1k cell stage – the start of zygotic transcription/organ development), (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; Thépot and Jerry, 2015; Walford and Lam, 1993).

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 were determined on a Sedgewick rafter counting cell based on the average of embryos counted from the five aliquots. Eggs were transferred to 500 L hatch tanks and stocked as per commercial practices (Schipp et al., 2007). For the collection of larvae for subsequent samplings at 12 hpf, 24 hph, and 48 hph, larvae in the hatch tanks were gently homogenized, while five aliquots of 40 mL were taken. To estimate the hatching rate and survival rates at 24 and 48 hph, the number of totals, live and dead larvae were determined 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 development stages of the eggs were verified before being preserved in 70% ethanol. Three distinct samples (i.e., ~300 eggs each) were taken as technical replicates to ensure an accurate representation of the family-specific spawning variability was measured 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.

5.3.4 Assessment of offspring phenotype

Eggs and embryos 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 embryo

morphological features were measured, including egg size (µm), egg roundness, oil droplet size (µm), and oil droplet roundness. For larvae, morphological features were measured, including total length (TL), yolk sac diameter (YS), and oil globule diameter (OG) at 12 hpf, and TL, OG, and eye diameter (ED) at 24 hph and 48 hph. The morphological features were measured using ImageJ analysis software (Version 1.41; http://rsbweb.nih.gov/ij/).

5.3.5 DNA extraction and genotyping

To allow individual parent–progeny relationships to be determined, each egg/larva was allocated to a single well in a 94 well plate for DNA extraction. DNA extraction and PCR amplification of microsatellites were performed according to Domingos et al. (2013). In brief, DNA of individual egg/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). For each spawning night and time point assessed, 94 larvae were sent to the Australian Genome Research Facility (AGRF), Melbourne, Australia, for genotyping.

5.3.6 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 the contribution of each male broodstock to the cohort of offspring and the total

number of half and full-sibling families within each spawning event and each night of each spawning event. A strict confidence level (CI) of 95% was utilized to determine the most likely parent pair assigned to offspring. Microsatellite data were formatted to GENPOP and Fstat file structure using GenAIEx 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), observed heterozygosity (H_o), 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 $Ned = \frac{N_d K_d - 1}{\left[K_d - 1 + \left(\frac{V_d}{K_d}\right)\right]}$ and sires $Nes = \frac{N_s K_s - 1}{\left[K_s - 1 + \left(\frac{V_s}{K_s}\right)\right]}$, and the effective population size $Ne = \frac{4 N_{ed} N_{es}}{N_{ed} + N_{es}}$ 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 $\Delta F = \frac{1}{2} N_e$ was calculated according to Falconer (1989).

5.3.7 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 K, fast motility, sperm viability, sperm DNA damage, paternal contribution at fertilization, and hatch time, were transformed with the logit function, and sperm velocities (i.e., VCL, VSL, and VAP) were transformed by $\log_{10}$ function. Pearson correlations were used to measure the relationship between different broodstock physical traits (i.e., BW, TL, and K), 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, K, 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 PC was determined based on the number of PCs that contributed to a pre-selected total of explained variation of 80%. Egg and larval morphometries were compared across spawns with one one-way ANOVA, followed by Tukey's multiple comparison test with statistical assumptions of homogeneity of variance and normality confirmed with a Levene's and Shapiro-Wilks normality test, respectively. When these assumptions were not met, a Kruskal-Wallis rank-sum test was performed, 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 $P < 0.05$.

5.4 Results

5.4.1 Characterizing baseline variation in broodstock condition and sperm quality

On average male barramundi broodstock were in good condition with BW 3.5 ± 0.2 kg, TL 70.1 ± 1.0 cm and K 1.004 ± 0.04. While the variation in physical traits between males were present, most male K ranged from 0.71 to 1.19, with the exception of one male with a K of 1.59 (Table 5.2). The milt characteristics across the broodstock were highly variable, with milt volume ranging from 3.5 to 69.7 µL (CV = 62.5%), sperm concentration ranging from 5.5 to 31.7 x 10^9 sperm/mL (CV = 36.9%) and total count ranging from 35.0 to 2023.3 x 10^6 sperm (CV = 82.2%; Table 5.2). Similarly, the proportion of motile spermatozoa varied greatly between individuals with total motility ranging from 13.4 to 70.1% (CV = 37.5%), progressive motility from 9.2 to 60.0% (CV = 45.5%), and fast motility from 2.4 to 46.1% (CV = 80.3%; Table 5.2). While sperm swimming trajectory parameters (i.e., LIN, STR, WOB, ALH, and BCF; Table 5.2) were relatively consistent between males (CV < 25%), measures of average sperm velocity, including VCL of 83.3 ± 4.5 µm/s, VSL of 47.0 ± 5.1 µm/s, and VAP of 72.4 ± 4.9 µm/s, showed higher variation (CV = 25.5%, CV = 50.6%, and CV = 31.6% respectively; Table 5.2). Of note, the male with the highest K had a low milt volume and sperm concentration, but had the highest sperm motility (i.e., TM: 70.1 ± 2.3%, PM: 60 ± 7.8%, and Fast: 46.1 ± 10.6%), sperm velocities

(i.e.,VCL: 152.9 ± 27.2 µm/s, VSL: 128.3 ± 24.3 µm/s, and VAP: 147.3 ± 29.2 µm/s), and the straightest swimming trajectory (i.e., 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; Table 5.2; Appendix H). Sperm viability across individuals was high overall, with only 15.6 ± 1.8% of the total spermatozoa population considered dead (Table 5.2). The integrity of sperm DNA was also high overall, with most individuals having less than ~1% DNA damage (median: 0.7%; Table 5.2), however, three males with sperm DNA damage levels of 19.8 ± 0.3%, 24.7 ± 0.1%, and 58.9 ± 0.9% were observed, resulting in a mean of 6.5 ± 2.9% and high total variation (CV = 207.7%; Table 5.2; Appendix H).

Table 5.2 Characteristics of captive-bred barramundi (*Lates calcarifer*, *n* = 22) broodstock physical traits, milt characteristics, and sperm quality parameters.

Parameter	Mean ± SEM	Median	Min	Max	CV (%)
Physical traits					
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)	731.5 ± 128.1	524.5	35.0	2,023.3	82.2
Sperm motility[†]					
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					
Dead (%)	15.6 ± 1.8	14.1	6.2	39.3	10.2
DNA-damaged (%)	6.5 ± 2.9	0.7	0.01	58.9	207.7

Data are displayed as mean ± standard error (SEM); 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). [†]see Appendix H for sperm motility data per fish.

5.4.2 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 (Table 5.3). Specifically, there was a negative correlation between BW and milt volume ($r(22) = -0.46$, $P < 0.05$), 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 5.3). Broodstock length was found, however, to be negatively correlated to sperm motility (TM, PM, and Fast; Table 5.3). K 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 positive correlation with Fast, VCL, VSL, and VAP (Table 5.3).

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 5.3). 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 5.3).

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 variables (i.e., sperm velocities and sperm integrity; Table 5.3). 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 5.3).

Table 5.3 Relationship between physical traits, milt characteristics, and sperm quality parameters for 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 condition factor K, Fast motility, sperm viability, sperm DNA damage and $\log_{10}$ transformed for VCL, VSL, and VAP. Abbreviations: BW, body weight; TL, total length; K, condition factor; 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, 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 statistical significance.

5.4.3 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 5.4). However, all spawns produced a viable batch of larvae without detectable physical deformities, except for night 1 from Tank A, which in association with a low fertilization rate (5.2 ± 1.0%) resulted in too few hatched larvae to determine an accurate hatch rate (< 0.001%; Table 5.4).

Table 5.4 The number (*n*) of females, the total number of eggs produced, egg production per kilogram, fertilization and hatching rates of barramundi (*Lates calcarifer*) for each breeding cohort and mass-spawning night.

	Tank A		**Tank B**		**Tank C**	
Parameter	**Night 1**	**Night 2**	**Night 1**	**Night 2**	**Night 1**	**Night 2**
Female (*n*)	4	4	4	4	3	3
Eggs produced (million)	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 (million 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 (SEM)

Across all spawning events, there were several general trends observed throughout embryonic and larval development. Progeny mortality occurred mainly between the fertilization stage and 24 h following hatching. An initial mortality event occurred during the embryonic development between assessment at 2.5 hpf and hatching, where on average ~25% of fertilized embryos did not continue to develop and/or hatch (Table 5.4). The second larval mortality event occurred over the next 24 h of larval development, where an additional ~25% of larvae were lost across all spawns except for Tank B (Table 5.5). The larval survival rate then stabilized across all spawns between 24 and 48 hph, with very few further mortalities observed (survival rate ≤ 90.9%; Table 5.5).

Table 5.5 Sequential and cumulative larval survival rates at 2.5 h post-fertilization (hpf) and 24 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 (SEM); Abbreviations: Sequential (Seq.); Cumulative (Cum.)

Egg size showed significant variation between spawns ranging from 705.7 ± 2.8 to 781.6 ± 2.1 µm (ANOVA, F(5, 294) = 122.7, $P < 0.001$; Table 5.6). However, egg roundness was consistent ($\bar{X}$ = 0.98 ± 0.001; Kruskal-Wallis, H(5) = 5.31, $P = 0.38$; Table 5.6). Similarly, oil droplet size varied significantly between spawns from 208.5 ± 2.4 to 256.5 ± 1.3 µm (Kruskal-Wallis, H(5) = 202.44, $P < 0.001$), while oil droplet roundness was also consistent ($\bar{X}$ = 0.97 ± 0.002; Kruskal-Wallis, H(5) = 9.58, $P = 0.088$; Table 5.6).

Table 5.6 Egg number and morphometry of barramundi (*Lates calcarifer*) for each breeding cohort and mass-spawning night.

	Tank A		**Tank B**		**Tank C**	
Parameter	**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 (SEM); *n*, the number of eggs measured. Different letters indicate significant differences ($P < 0.001$).

The total length of hatched larvae varied significantly between spawns (ANOVA, F(4, 95) = 35.62, $P < 0.001$) and ranged from 1,626 ± 38 to 2,069 ± 23 µm (Table 5.7). The total length of larvae increased

by an average of 590 µm from hatch to 24 hph; however, the differences between spawns remained significant (Kruskal-Wallis, H(4) = 13.37, $P = 0.0096$). The yolk sac with an initial size ranging from 695 ± 7 to 901 ± 11 µm (Kruskal-Wallis, H(4) = 78.65, $P < 0.001$) was absorbed completely at 24 hph (Table 5.7). Total length plateaued between 24 and 48 hph, yet, total length variance at 48 hph diverged significantly between spawns (Kruskal-Wallis, H(4) = 48.26, $P < 0.001$). Once eyes of larvae were fully formed at 24 hph, average eye size remained consistent between 24 and 48 hph (~233 µm), but differed significantly between spawns for both time points (24 hph, ANOVA, F(4, 36.99) = 6.98, $P = 0.0003$; 48 hph, ANOVA, F(4, 77) = 4.85, $P = 0.002$; Table 5.7).

Table 5.7 Larva morphometry of barramundi (*Lates calcarifer*) at hatch time and 24 and 48 h post-hatch (hph) for each breeding cohort and mass-spawning night, including total length (TL), yolk sac feret diameter (YS), eye diameter (ED), and oil globule diameter (OG).

		Tank A		**Tank B**		**Tank C**	
Time	**Parameter**	**Night 1**	**Night 2**	**Night 1**	**Night 2**	**Night 1**	**Night 2**
Hatch	TL (µm)	NA	1,626 ± 38^{c}	1,777 ± 18^{b}	1,650 ± 17^{c}	1,893 ± 46^{b}	2,069 ± 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	2,369 ± 12^{ab}	2,431 ± 36^{a}	2,440 ± 32^{ab}	2,347 ± 16^{b}	2,382 ± 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	2,443 ± 35^{abc}	2,306 ± 24^{bc}	2,414 ± 21^{ab}	2,480 ± 15^{a}	2,199 ± 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 (SEM) for $n = 20$ except for Tank A – night 2 at 24 hph $n = 10$ and at 48 hph $n = 2$. hph.

5.4.4 Parental contribution

The overall participation of broodstock in the resultant progeny cohort was high across the three spawning events. All males were found to have contributed at least once across the two consecutive spawning nights. Specifically, in Tank A, seven out of eight males were detected to have contributed in night 1, while all males were detected as participating parents to progeny from the night 2 spawn (Figure 5.1; Appendix I). All males' contribution was detected in Tanks B and C for night 1 and night 2 spawns

(Figure 5.1; Appendix I). The number of offspring successfully genotyped and assigned to a pair parent at 2.5 hpf and 24 hph for each mass-spawning night was high on average (Appendix I). The proportion of male contribution was highly skewed, with males dominating paternal contribution up to 54.3% in Tank C (Figure 5.1; Appendix J).

Female contribution to the resultant progeny cohort was inconsistent. Female 1 from Tank A did not participate in the spawning from either night of spawning, while Female 6 and 7 from Tank B and Female 10 from Tank C only contributed one night of spawning each (Figure 5.1; Appendix I). Females that dominated the spawning event could contribute as much as 95.6% of the maternal contribution, such as in Tank A (Figure 5.1; Appendix J). The number of families detected from nights 1 and 2 at 2.5 hpf and 24 hph was about half the number of potential families that could have been generated with an even parental contribution (Figure 5.1; Appendix I).

5.4.5 Genetic diversity

The broodstock cohort from Tank A showed the highest average allele number (3.5/locus), expected (0.502), and observed (0.536) heterozygosity, followed by the cohorts from Tanks B (3.19/locus, 0.504 and 0.542) and C (3.06/locus, 0.481 and 0.543; Table 5.8). The F_{is} value for the three broodstock cohorts was negative and significantly larger than 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 (Appendix K). The F_{is} values for offspring across all spawns were negative and significantly larger than 0 ($P < 0.05$; Table 5.8).

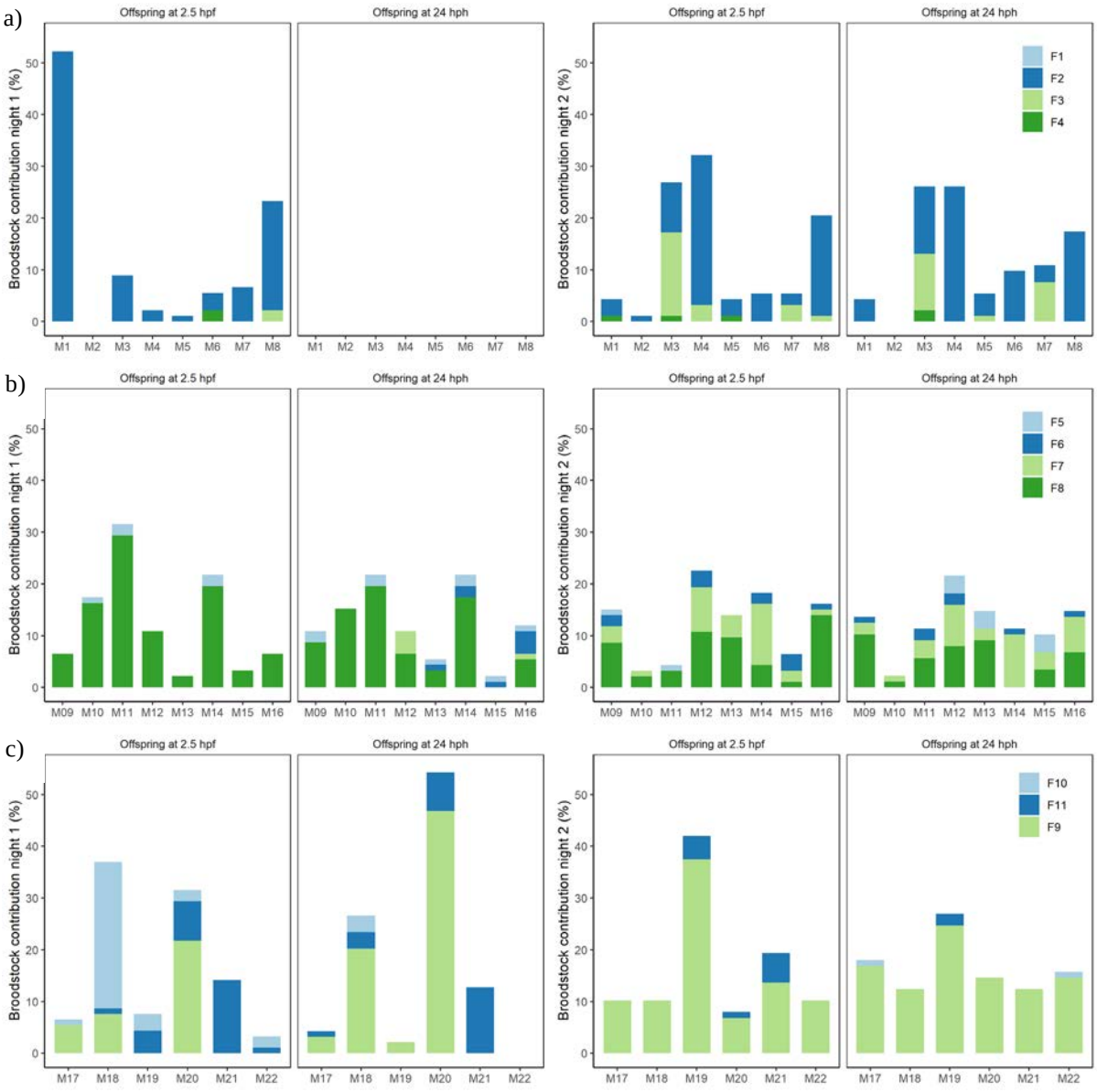

Figure 5.1 Proportion of barramundi (*Lates calcarifer*) broodstock contribution displayed for night 1 and 2 at 2.5 h post-fertilization (hpf) and 24 h post-hatch (hph) from Tanks A (n = 12), B (n = 12), and C (n = 9). Each male contribution is displayed on the x-axis, while female contribution is shown in the stacked bar plot.

Table 5.8 Measures of genetic diversity of barramundi (*Lates calcarifer*) broodstock and offspring for Tanks A, B, and C; Sample size (N_c), number of alleles (k), average observed (H_o) and expected (H_e) heterozygosity, allelic richness (A_r), average inbreeding coefficient (F_{is}), effective population size (N_e), rate of inbreeding (ΔF), and N_e/N_c ratio. Spawns A and B represent the first and second night of spawning, respectively.

	Tank A					**Tank B**					**Tank C**				
	Broodstock	**Offspring**				**Broodstock**	**Offspring**				**Broodstock**	**Offspring**			
		Night 1		**Night 2**			**Night 1**		**Night 2**			**Night 1**		**Night 2**	
		at 2.5 hpf	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**		**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**		**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 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*	-0.13*	-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 ($P < 0.001$)

5.4.6 Effect of male reproductive condition on spawning performance and early embryonic development in barramundi

Overall the linear relationships between each male-sperm quality parameters and paternal contribution observed at 2.5 hpf and 24 hph were moderate. Paternal contribution at fertilization for night 1 showed a positive correlation with sperm concentration ($r(22) = 0.45$, $P < 0.05$), whereas no significant relationships were found between sperm quality parameters and paternal contribution for night 2. Paternal contribution of larvae for night 1 was, however, negatively correlated to K ($r(22) = -0.62$, $P < 0.01$), VSL ($r(22) = -0.44$, $P < 0.05$), VAP ($r(22) = -0.42$, $P < 0.05$), sperm DNA damage ($r(22) = -0.67$, $P < 0.001$), while paternal contribution of larvae for night 2 was negatively correlated to K ($r(22) = -0.44$, $P < .05$), VCL ($r(22) = -0.46$, $P < 0.05$), and VAP ($r(22) = -0.43$, $P < 0.05$).

The PCA conducted using all males showed a total of five first principal components accounted for 81.8% of the total variance present in the study. Of these, PC1 and PC2 have accounted for 33.01% and 17.09%, respectively (Figure 5.2a). The first component (PC1) was significantly positively correlated with milt volume ($r(22) = 0.64$, $P < 0.01$), sperm concentration ($r(22) = 0.77$, $P < 0.0001$), paternal contribution at fertilization for night 2 ($r(22) = 0.64$, $P < 0.01$), and paternal contribution of larvae for night 1 ($r(22) = 0.64$, $P < 0.01$) and negatively correlated with BW ($r(22) = -0.56$, $P < 0.01$), K ($r(22) = -0.75$, $P < 0.0001$), VCL ($r(22) = -0.78$, $P < 0.001$), VSL ($r(22) = -0.86$, $P < 0.001$), VAP ($r(22) = -0.82$, $P < 0.001$), and sperm DNA damage ($r(22) = -0.73$, $P < 0.001$). This suggests that these 10 variables co-vary, 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 to 24 hph. The second component (PC2) was significantly positively correlated with BW ($r(22) =0 .50$, $P < 0.05$), TL ($r(22) = 0.75$, $P < 0.0001$), sperm DNA damage ($r(22) = 0.49$, $P < 0.05$), paternal contribution at fertilization for night 2 ($r(22) = 0.48$, $P < 0.05$), and paternal contribution of larvae for night 2 ($r(22) = 0.63$, $P < 0.01$), while negatively correlated to TM ($r(22) = -0.50$, $P < 0.01$), VCL ($r(22) = -0.43$, $P < 0.01$), and ALH ($r(22) = -0.69$, $P < 0.001$). The second component suggests that in general larger males have a lower proportion of motile spermatozoa, higher sperm DNA damage,

and reduced VCL and ALH during swimming. They might also 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 milt volume ($r(22) = 0.46$, $P < 0.05$), TM ($r(22) = 0.56$, $P < 0.01$), BCF ($r(22) = 0.56$, $P < 0.01$), and sperm viability ($r(22) = 0.83$, $P < 0.0001$), suggesting that larger milt samples at collection in general have an higher TM and BCF. The fourth component (PC4; 11.2% of total variance) was negatively correlated to BW ($r(22) = -0.46$, $P < 0.05$), TL ($r(22) = -0.56$, $P < 0.05$), and paternal contribution at fertilization for night 1 ($r(22) = -0.66$, $P < 0.001$), and positively correlated to paternal contribution at fertilization for night 2 ($r(22) = 0.58$, $P < 0.01$) and paternal contribution of larvae for night 2 ($r(22) = 0.49$, $P < 0.05$), meaning that smaller males had an higher contribution during night 2 compared to the night 1 spawning. The fifth component (PC5; 7.4% of total variance) is primarily correlated with BCF ($r(22) = -0.56$, $P < 0.01$) and paternal contribution of larvae 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 (Figure 5.2b-c). PCA using individuals from Tank A showed a PC1 and a PC2 explaining 41.76% and 26.28%, from Tank B showed 31.09% and 27.28%, and from Tank C showed 42.75% and 25.14% of the total variance (Figure 5.2).

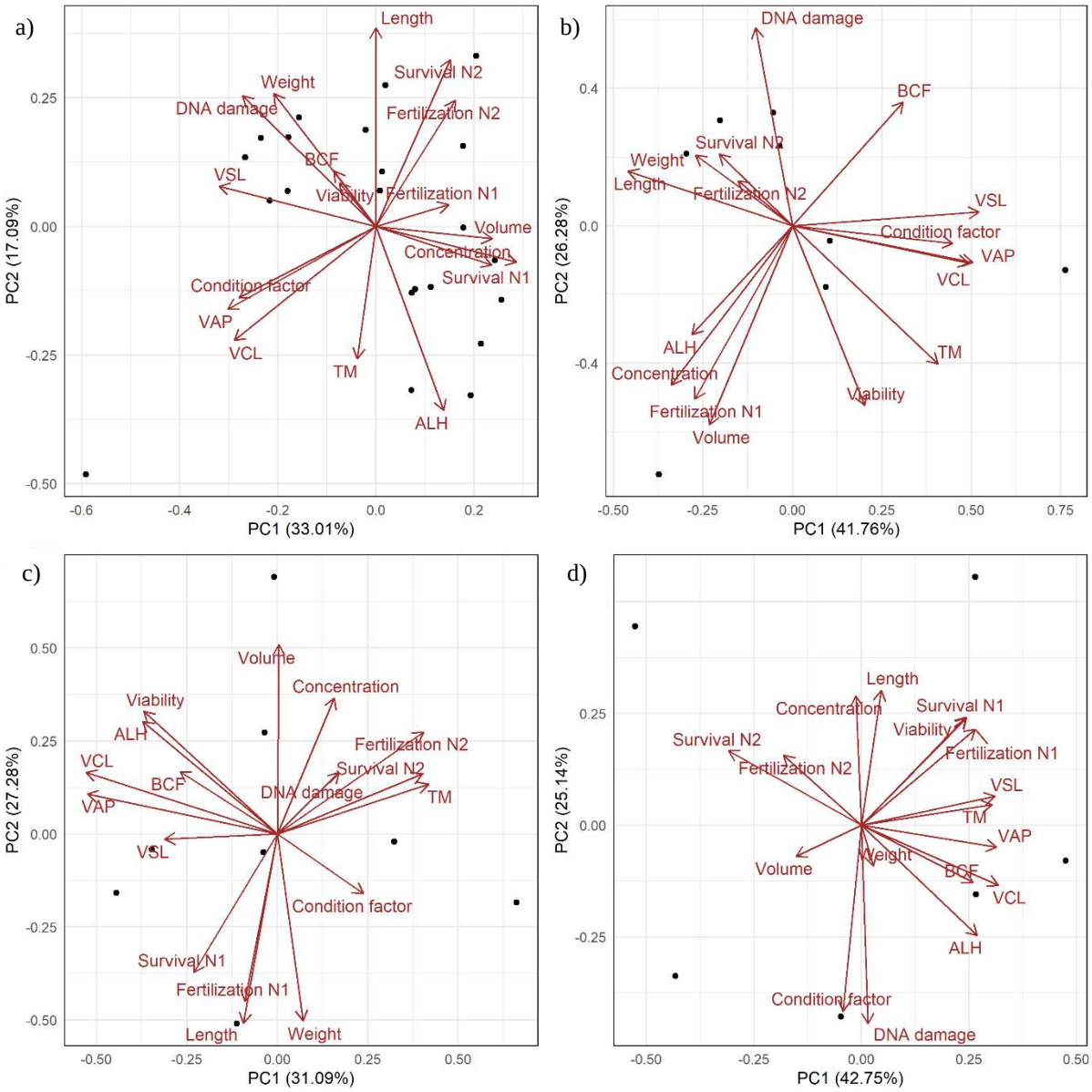

Figure 5.2 Principal component analysis (PCA) of male barramundi (*Lates calcarifer*) broodstock reproductive condition and performance during a mass-spawning event, including two consecutive spawning nights (N1 and N2). (a) PCA including all males (n = 22). (b) PCA including males (n = 8) from Tank A. (c) PCA including males (n = 8) from Tank B. (d) PCA including males (n = 6) from Tank C. Dots represent individual males. The proportion of variance captured is given as a percentage for the first and second principal components (PC1 and PC2).

5.5 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 male-sperm quality was high, indicating the presence of males at various levels of the reproductive condition, direct relationships between male-sperm quality parameters and paternal contribution of newly fertilized embryos were weak for most parameters, except for sperm concentration. However, a strong negative relationship between sperm DNA damage and paternal contribution was observed at the larval stage. While sperm quality was identified to affect paternal contribution, other factors, including artificial spawning induction, spawning behavior, social hierarchy, and tank environment, may have played a larger role in the outcomes of mass spawns than individual male reproductive conditions. These data offer the first insight into the dynamics influencing the outcome of mass-spawning events and a baseline for further research in barramundi breeding.

In this study, sperm quality was associated with broodstock's physical condition. The broodstock condition factor was shown to directly correlate with sperm motility and milt characteristics. For instance, males with a high condition were found to have higher sperm motility and a reduced sperm concentration. These are characteristics of males at an advanced stage of spermiation (Schulz et al., 2010). The advanced spermiation stage 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 alteration of the seminal plasma composition allows immature spermatozoa to acquire motile capability (Schulz et al., 2010). However, while males with higher condition factors showed signs of advanced spermiation, the milt volume collected from these males was less than from males with highly concentrated spermatozoa and low motility. This divergence is most likely the result of the cannulation method (Ohta et al., 1997; Suquet et al., 2000). In contrast to milt volume collected by hand stripping, the volume collected by cannula is not a finite measure of milt volume, as variation in the time, pressure, and intensity, exercised by the technician to extract milt samples might have varied between cannulations.

The use of advanced sperm function assessments revealed no association between sperm motility and fertilization success. Instead, broodstock having highly concentrated and low motile spermatozoa had a higher contribution during the spawning. This outcome is in contrast with most studies. Sperm motility is usually strongly associated with fertilization capabilities (Beausoleil et al., 2012; Fauvel et al., 1999; Gage et al., 2004; Linhart et al., 2005). This outcome may have resulted from the LHRHa stimulation performed on males after sperm quality assessment to trigger the spawning event. The LHRHa injection may have supported milt hydration in males with a low reproductive condition (Mylonas et al., 2017). In contrast, males already at an advanced stage of spermiation may have been hyperstimulated by the additional exogenous LHRHa injection, impeding their spawning response. While previous studies using barramundi observed skewed paternal contribution during mass-spawning events without injecting males, the hormonal stimulation of males performed in this study did not seem to reduce the variation between male contributions. To date, the effect of LHRHa injection on barramundi male-sperm quality is unknown and is only used by commercial hatcheries to improve male participation during spawning. Further investigations are, therefore, necessary to quantify the effect of LHRHa on male-sperm quality and investigate the use of a dose-dependent LHRHa injection on male pre-spawning conditions. These studies may provide valuable recommendations to optimize barramundi breeding practices and spawning outcomes.

Although LHRHa hyperstimulation of mature testes may be one cause of their reduced contribution during mass-spawning events, high levels of sperm DNA damage found in some barramundi broodstock males could be another cause of skewed contribution. In this study, sperm DNA damage has been strongly associated with males of higher weight and condition factor, lower sperm concentration, and spermatozoa with higher velocity and linear swimming pathway, which are characteristic of males in an advanced stage of spermiation. While the cause of DNA damage is unknown, the presence of a high level of sperm DNA damage in barramundi broodstock, reaching up to 58.9% in this study, is intriguing and abnormal. Similarly, high levels of DNA damage have been observed previously in barramundi broodstock with average sperm DNA damage of 43.5 ± 6.0% (Chapter 3; Marc et al., 2021). A high level of sperm DNA damage is usually reported in spermatozoa exposed to endocrine-disrupting

chemicals (e.g., oestrogens; Rempel et al., 2009, 2006), genotoxic agents (Ciereszko et al., 2005; Dietrich et al., 2005; Hulak et al., 2013; Santos et al., 2013), and cryopreservation (Cabrita et al., 2005b; Figueroa et al., 2020; Pérez-Cerezales et al., 2009; Zilli et al., 2003). However, in mammals, it has been shown that mature spermatozoa are susceptible to damage by reactive oxygen species (Aitken et al., 2013) and contain apoptotic-like mechanisms, which they acquired during milt hydration (Gawecka et al., 2015). It is also known that once spermatozoa reach maturity, they are unable to repair their DNA (Olsen et al., 2005) and rely solely on seminal plasma antioxidants to neutralize oxidative compounds (Figueroa et al., 2020). Moreover, in teleosts, it has been reported that spermatozoa stored for an extended period in the sperm duct were subject to *in situ* degeneration, starting with the progressive disaggregation of the chromatin into small filaments (Billard and Takashima, 1983; Cattelan and Gasparini, 2021). The outcome of these previous studies combined with our observations leads to corroboration of the hypothesis that the cause of high levels of sperm DNA damage solely observed in mature spermatozoa from males at an advanced stage of spermiation might be the result of oxidative stress. This stress may be induced by factors linked to current hatchery activities and rearing practices, which warrants further investigation.

The presence of DNA damage in spermatozoa of males at an advanced stage of spermiation was also negatively associated with the survival rate of offspring. Embryonic losses occurring during early developmental stages has been linked in several studies to sperm DNA damage (Depincé et al., 2020; Figueroa et al., 2020) and paternal effects on early life-history traits (Butts et al., 2009; Butts and Litvak, 2007ab; Rideout et al., 2004; Saillant et al., 2001; Siddique et al., 2017). The specificity of sperm DNA damage to induce malformation and mortality in early larval development has been linked to paternal genes supporting embryonic growth (e.g., insulin growth factor (*Igf*), growth factor (*Gh*), and insulin (*Ins*)) emplacement on the chromatin (Cartón-García et al., 2013; Pérez-Cerezales et al., 2011). Paternal genes supporting embryonic growth are compartmentalized in less compacted chromatin arrays to be readily accessed for early transcription (Figueroa et al., 2020). As a trade-off, genes located in less compacted arrays are more sensitive to oxidative damage leading to alterations in the transcription of

these genes and ultimately to developmental arrest (Figueroa et al., 2020). In turn, our data provide the first evidence of the paternal effect on early life-history traits in barramundi.

Although DNA damage in spermatozoa of mature males affected early larval survival on the first spawning night, offspring sired by the same mature males on the second night were not affected. This difference between nights might be due to the recrudescence of new spermatids that matured within the 24 h following the first spawn, allowing newly matured spermatozoa with high DNA integrity to fertilize eggs and generate healthy eggs larvae the second night. Moreover, the reduction of sperm DNA damage seemed to affect skewed paternity as the broodstock cohort from Tank B had the lowest sperm DNA damage level (mean: 0.07 ± 0.03%) compared to Tanks A (mean: 15.67 ± 6.85%) and C (mean: 2.64 ± 1.18%), and had the lowest paternal skew. A similar trend can be observed with the reduction of paternal skew in the second spawning night when compared to the first night in Tanks A and C. While additional spawns are required to quantify more accurately the relationship between skewed paternity and sperm DNA damage, our data support the hypothesis that the level of sperm DNA damage linked to the extended storage of mature spermatozoa in the sperm duct and not to male fertility *per se*. Consequently, studying the life cycle of barramundi spermatozoa from germ cells to maturity and investigating factors inducing DNA damage of mature spermatozoa could provide essential improvements to current commercial hatchery practices. Ultimately, these recommendations will optimize the sperm integrity of males before the induction of a spawning event and potentially lead to a reduction in skewed paternity.

While male variation in breeding conditions and sperm quality parameters such as sperm DNA damage have been demonstrated to affect spawning outcome, the lack of direct correlation between sperm motility parameters and fertilization success indicates other factors are at play. The divergence in parameters driving spawning outcome between tanks and the presence of highly skewed maternal contributions indicate the influence of additional factors, such as stress, tank environment, spawning behavior, and social hierarchy on individual male-sperm quality. 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 female and/or release (unpublished data). The hormonal-inducing spawning procedure performed before the spawning event, including draining the tank, netting, anaesthesia, cannulating the broodstock, and the LHRHa injection, may have generated disturbance to broodstock hierarchies and hence mating behaviors. The effect of group hierarchy has also been a major factor in spawning synchronization and skewed paternity (Bekkevold et al., 2002; Coe et al., 2008). Moreover, research on zebrafish has also shown that social hierarchy affects individual sperm quality and ultimately siring performance (Zajitschek et al., 2017, 2014). Male reproductive behavioral traits and social status are passed on to the offspring through sperm-mediated genetic and epigenetic effects of sperm fitness (Zajitschek et al., 2017, 2014). Hitherto, the complex underlying mechanisms of the inherited paternal effects caused by environmental and social cues passed to the offspring may play a subsequent role in explaining the variation observed in mass spawns with barramundi.

Although additional research investigating the causes of skewed maternal and paternal contribution may provide valuable knowledge, these undertakings might not be sufficient to limit the variability of spawning outcome and gain greater control over broodstock contribution to the next generation of offspring. While the genetic diversity of broodstock may be high enough, the resulting negative average of the F_{is} value observed in broodstock cohorts attests to the history of skewed contribution in the domestic line. Moreover, the inbreeding coefficient resulting from the spawn performed using the current breeding practices ranged, in our study, from 5.9% to 17.3%, which far exceeded the recommended average of 0.5% for a population to be used for a selective breeding program. The development of advanced breeding technology for barramundi is in its infancy; however, a shift to artificial fertilization might be necessary to encompass challenges linked to the mass-spawning strategy for selective breeding programs (Domingos et al., 2013; Loughnan et al., 2013; Macbeth and Palmer, 2011; Robinson et al., 2010).

5.6 Conclusion

This study is the first to explore interactions between male barramundi reproductive condition – sperm quality and paternal contribution during mass-spawning events. While our data highlight the occurrence of abnormal levels of sperm DNA damage in captive-bred broodstock and the importance of the paternal genetic contribution to the early larval developmental stages, further investigations are warranted to improve breeding practices in barramundi further.

Chapter 6. General discussion

6.1 Significance and major outcomes

Barramundi (*Lates calcarifer*) is an aquaculture species of commercial importance farmed throughout Australia, Southeast Asia, and increasingly globally (Jerry, 2013). Over the last decade, Australian farmed barramundi has grown in popularity among consumers due to its high flesh quality (i.e., boneless fillet; Schipp et al., 2007). Despite barramundi aquaculture being the fastest growing aquaculture sector in Australia with an annual growth of 14% p.a., the demand for Australian farmed barramundi is currently outstripping production due to a constantly growing demand from international and domestic markets. This excess demand for barramundi is driving investment into the growth of the Australian barramundi industry to increase production volume. As such, according to the 2019 annual report of the Australian Barramundi Farmers Association (ABFA), the Australian barramundi industry is poised to expand its infrastructure and bring innovation to its farming practices to increase barramundi production from 9,000 to 25,000 tonnes by 2025 while maintaining high production standards and product quality (FRDC, 2020). Although large infrastructure investments are underway to build new high-tech grow-out facilities and expand existing ponds and sea cage infrastructure, optimizing farming procedures, such as feeding, harvest, and hatchery, is key to unlocking production. One of the most promising innovations in the field is the use of barramundi genetically selected for traits of commercial interest and increased productivity (e.g., growth, disease resistance, and flesh color) to realize the full potential of the species in aquaculture (Domingos et al., 2013; Gjedrem et al., 2012; Gjedrem and Robinson, 2010; Loughnan et al., 2013; Macbeth and Palmer, 2011; Robinson et al., 2010; Robinson and Jerry, 2009).

Several selective breeding programs for barramundi have been instigated. However, the complex barramundi life-cycle and mass-spawning breeding strategy often render genetic selection difficult and onerous due to the lack of control of the spawning outcomes (Macbeth and Palmer, 2011; Robinson et al., 2010). In previous studies assessing barramundi mass-spawning, highly skewed paternal

contribution levels were observed during mass-spawning events (Domingos et al., 2014, 2013; Frost et al., 2006; Liu et al., 2012; Loughnan et al., 2013; Wang et al., 2008). Despite having an initially large number of broodstock with high genetic diversity in the breeding cohort, the effective number of broodstock contributing to the next generation was low and resulted in an important loss in genetic diversity in the subsequent generations produced. Consequently, the inbreeding rates of progeny ranged from 3 to 17.9%, which far exceeded the recommended average of 0.5% for conducting a breeding program (Domingos et al., 2014; Loughnan et al., 2013). Moreover, in all studies assessing mass-spawning events in barramundi, highly skewed paternal contribution patterns were consistently observed, suggesting fertility issues are potentially present in male barramundi reared in a captive environment (Domingos et al., 2014, 2013; Frost et al., 2006; Liu et al., 2012; Loughnan et al., 2013; Wang et al., 2008). Accordingly, the investigation of the fertility status of male barramundi was warranted to understand the link between male fertility and their contribution during spawning.

Reproductive disorders and unpredictable spawning outcomes are common problems for many aquaculture species (Migaud et al., 2013; Mylonas et al., 2010). However, leading aquaculture industries such as those based on Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss)*, European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), and turbot (*Scophthalmus maximus*) have used advanced reproductive technologies (ARTs) to gain control over the breeding process and enable the realization of effective genetic gain in selective breeding programs (Gjedrem and Robinson, 2010; Janssen et al., 2015; Migaud et al., 2013; Mylonas et al., 2010). ARTs include gamete quality assessments, gamete handling procedures, gamete storage protocols for both the short- (i.e., chilled storage) and long-term (i.e., cryopreservation), and artificial fertilization. Using these techniques, it is possible to (i) assess broodstock gamete quality, (ii) select and collect gametes from the best broodstock, (iii) fertilize eggs when required with full control of the number of offspring generated, and (iv) control the genetic heritage transmitted to the offspring, and the timing of fertilization (Mylonas et al., 2010).

In Chapter 2, to develop these techniques for barramundi, an initial review of teleost reproductive biology was undertaken. This review was mainly aimed at understanding how barramundi differs from other species currently implementing ARTs. The outcome of the review revealed that there are two different structural types of testis in teleosts, classified either as anastomosing tubular or lobular testis. In anastomosing tubular testis, seminiferous tubules are interconnected, and spermatogonia follow an unrestricted spatial distribution in the germinal compartment. In contrast, in lobular testis, seminiferous tubules connect individually to the albuginea tunica, and spermatogonial distribution can be of the restricted, semi-restricted, or unrestricted type (Knapp and Carlisle, 2011). Interestingly, most species bred using ARTs have anastomosing tubular testes. For this type of testis, spermatogonia develop and mature synchronously within the testis following distinct environmental changes (e.g., photoperiod) associated with the four-season cycle that allows synchronous spawning over a specific period (e.g., autumn for rainbow trout; Grier, 1993) and also to collect mature spermatozoa in large quantities. Sperm production in barramundi, however, occurs in lobular testes, which seem to follow a semi-restricted spermatogonial distribution. For this type of testis, spermatogonia develop and mature in cysts and migrate to the spermatic ducts by appositional growth. As a result, smaller amounts of spermatozoa are consistently released into the sperm duct, permitting barramundi to breed over an extended time (e.g., ~6 months breeding season), following the wet-dry seasonal cycle, characteristic of tropical regions. These fundamental differences in sperm production, highlighted in Chapter 2, warranted the optimization of current methodologies developed mostly for temperate teleosts to assess fertility and sperm quality in barramundi.

In Chapter 2, it was also highlighted the importance of cues (e.g., photoperiod, temperature, and salinity) for driving fish reproductive conditioning and sperm quality varied according to a species' geographic origins (i.e., temperate, subtropical, and tropical) and environments (i.e., fresh water, brackish water, and salt water). The outcome of studies assessing these environmental cues showed a wide range of responses within and between the species assessed, emphasizing the requirement for a species-specific approach to assessing barramundi fertility.

Finally, in Chapter 2, the review of the methods used to assess reproductive condition/sperm quality showed a considerable variation in approaches between species/research groups. The description of the experimental designs used to carry out these assessments (e.g., pooled *vs.* individual assessment, the number of replicates and sub-replicates performed, motility thresholds for immotile/motile sperm and motile subpopulations) was in many cases lacking, irrespective of the type of methodology used, limiting the interpretation of the data. Despite the availability of semi/automated methodologies (e.g., cell counters, CASA, and flow cytometry), the use of manual methods to assess sperm concentration (e.g., haemocytometer), sperm motility (e.g., visual scoring from 0 to 5), and sperm integrity (e.g., visual assessment using fluorescent microscopy) were still common. However, these manual methods are time-consuming, labor-intensive, and provide a limited overview of the sample quality (< 200 sperm assessed), limiting their use to small-scale assessments only. Therefore, due to fundamental physiological differences between species and a lack of standards across studies, this review highlighted an overall need for developing trials using a stepwise approach to validate factors known to influence sperm quality and ensure the development of a reliable and scalable procedure for use with barramundi. Specifically, three research priorities emerged to develop sperm quality analysis in barramundi. The first priority was to minimize the time spent on the analysis by focusing on optimizing technologies, allowing for a simple, high-throughput, and automated analysis. The second priority was to provide a standardized procedure to generate robust and unbiased datasets and enhance reproducibility to facilitate longitudinal studies. The third priority was to optimize the sperm handling and storage methodology for barramundi to ensure that procedures performed between the time of sperm collection and the assessment of the sperm quality were not adversely affecting the results. Ultimately, this approach and experimental design provide a structure to validate the use of sperm quality assessments for other established and new aquaculture species.

In Chapter 3, research focused on addressing priorities 1 and 2. Specifically, advanced sperm function assessments were validated using a high-throughput methodology to assess barramundi sperm motility and integrity. Computer-assisted sperm analysis (CASA), commonly used in human and livestock fertility centers, was optimized to detect barramundi spermatozoa. CASA can now be used to provide

a rapid and reliable quantitative assessment of sperm motility in barramundi. CASA detection thresholds were adjusted based on morphological measurements of 200 spermatozoa to create a detection profile and enable automated recognition of barramundi spermatozoa. Counts generated automatically from 90 videos recording barramundi spermatozoa with CASA were compared to the manual counts of spermatozoa to validate the accuracy of the CASA-derived barramundi sperm profile. Once the profile was validated, a standard dilution factor was determined, comparing the sperm concentration using CASA with sperm concentration obtained manually using the haemocytometer as a control. The best agreement between both methods was found when samples were diluted 1:1000, allowing for the simultaneous assessment of 35 to 250 sperm per video/field of view. Moreover, the accuracy of the CASA sperm concentration estimates improved compared to the manual method when four or more fields of view were recorded for each replicate, reducing variability between measurements to less than 7.5%. Finally, there was no effect of motility on sperm concentration estimates. The initial steps to validate CASA for barramundi sperm assessment were tedious and labor-intensive but revealed necessary to develop a reliable procedure. During the development of the CASA's barramundi sperm profile, it was found that small errors in the detection of barramundi spermatozoa had large repercussions on the final sperm concentration and motility values, as variables such as total motility, progressive motility, velocity parameters (i.e., VSL, VCL, VAP, LIN, STR, WOB, etc..) rely on the total number of spermatozoa present in the field of view to determine proportions and averages. Additional efforts were made during the optimization phase to validate key parameters influencing sperm motility estimates, including thresholds for immotile/motile sperm and motile subpopulations. Specifically, VCL $\geq$ 15 μm/s was determined using the 95% threshold cutoff method when recording motility of non-activated spermatozoa subject to artefactual motility caused by the Leja chamber capillary action to identify the sperm motile class.

In Chapter 3, the sperm viability and DNA integrity assays were the first molecular assays performed on barramundi spermatozoa. The optimization of both dual staining procedures using flow cytometry instead of fluorescent microscopy removed the necessity to count spermatozoa manually and facilitate rapid assessment of 100,000 cells while providing a reliable and scalable methodology. The validation

of both assays relied mainly on generating a usable positive control by optimizing incubation time, temperature, and intensity of the stressors and assessing the number of spermatozoa lost during the washing process to ensure sufficient spermatozoa remained at the end of the procedure. In conjunction with the data generated through optimizing sperm quality assays, a first quantitative sperm profile emerged for barramundi. These assays highlighted a dramatic reduction in sperm volume and motility compared to data reported from previous studies assessing sperm samples from wild-caught barramundi (Leung, 1987; Palmer et al., 1993). In addition, high levels of DNA damage in spermatozoa from many of the captive-bred male barramundi were found. However, at this stage, the comparison of sperm quality metrics from Chapter 3 and those from previous studies on sperm samples from wild-caught barramundi remains difficult due to the difference in methodologies used to assess sperm quality (i.e., visual estimation *vs.* automated motility assessment), collection (i.e., stripping *vs.* cannulation), and post-collection handling (e.g., chilled-storage time, non-activating medium (NAM) formulation). Moreover, while the accuracy of the sperm quality assessment was validated, the effect of the sperm handling method used in Chapter 3 remained to be assessed to ensure that it did not artificially induce damages to the sperm samples.

In Chapter 4, research focused on addressing priority 3 to optimize the sperm handling and storage methodology to ensure reliable collection and short-term storage for barramundi spermatozoa. During the initial optimization phase of sperm quality assessments, it was found that barramundi spermatozoa collected at the aquaculture facility could not withstand the handling procedure used previously by Palmer et al. (1993). In this chapter, it was found that the low osmolality of the NAM (i.e., Marine Ringer's solution at 260 mOsm; MRS260) was responsible for the high sperm lysis observed shortly after collection and that the increase of the osmolality to 400 mOsm (MRS400) was able to improve sperm viability after 1 h incubation in the NAM from 40% to 80%. The osmolality-adjusted NAM allowed to optimize and validate sperm quality assays in Chapter 3; however, motility of spermatozoa seemed artificially inhibited with only an average of 25% motile spermatozoa across sperm samples assessed. While the low proportion of sperm motility could either be a normal and natural characteristic of sperm production in barramundi or due to a lack of sperm maturation in captivity (e.g., a lack of

exposure to steroid hormones in the sperm duct), the ionic composition of NAM has also been shown to affect sperm motility in other species. Biochemical analysis performed on blood plasma and seminal plasma from saltwater barramundi demonstrated that increasing the osmolality of NAM from 265 mOsm/kg to 400 mOsm/kg was necessary to mimic the natural osmolality level of seminal plasma. This study led to further investigations into the role of Na^+, K^+, and pH with regard to sperm motility. The results revealed that MRS400 had an inhibiting effect on sperm motility due to the dissociation of the $NaHCO_3$ buffer into CO_2 at higher pH levels ($\leq$ 7.4). The replacement of the $NaHCO_3$ by HEPES, an inorganic buffer, permitted the retention of sperm motility after 24 h incubation in NAM when adjusted to 7.4 and 7.8, mimicking the natural seminal plasma range. Moreover, it was found that barramundi sperm motility was Na^+ dependent and demonstrated higher performance with a lower K^+ concentration (5 mM) than naturally occurring in the seminal plasma (~35 mM). With the improvement made in the formulation of NAM, barramundi spermatozoa can now be reliably stored for up to 24 h without significant degradation and will remain motile for up to 72 h. This research is the first of its kind reported for barramundi, providing the first biochemical profile of seminal plasma, insights into bio-mechanisms regulating sperm motility, and an optimized procedure to collect and store barramundi spermatozoa reliably. While the knowledge gained provides the foundation for future improvement of NAM formulation and development for a long-term storage procedure (i.e., cryopreservation), the ability to maintain sperm alive and motile longer than the initial 30 min opens the way to the development of artificial fertilization for barramundi.

In Chapter 5, an investigation of male barramundi fertility and its contribution to progeny post-mass-spawning events were examined. This final data chapter resulted from validating the sperm quality and handling methodologies in Chapter 3 and Chapter 4. In this chapter, it was found that male barramundi broodstock were not all in the same reproductive condition stage. Although male barramundi were held under the same broodstock conditioning environment (i.e., 30 °C water temperature, 30 ppt salinity, 16 h light: 8 h dark cycle, and fed a high-quality diet), most males were identified to be in the early stage of spermiation (i.e., low body condition factor, high sperm concentration, and low sperm motility), and were considered in low reproductive conditions. Only a few individuals were identified to be in the

advanced stage of spermiation (i.e., ready to spawn, ripe gonads, and high motile spermatozoa) and were considered in high reproductive conditions. Interestingly, a strong relationship was found between individuals with high reproductive conditions and sperm DNA damage. Levels of sperm DNA damage observed from these males in high reproductive conditions were of a similar range to sperm DNA damage levels recorded in Chapter 3. However, comparison between both chapters is limited to DNA integrity as different males were used to validating the sperm motility methodology in this chapter. While it is not possible to retrospectively determine the reproductive status of the males associated with these high sperm DNA damage levels in Chapter 3, it is indicative that sperm DNA damage could be a recurrent issue in hatcheries.

In this chapter, it was also found that sperm DNA damage was directly correlated with larval survival rate, demonstrating for the first time a potential paternal effect on the early larval development of barramundi embryos. This finding supports existing evidence that male genetic and epigenetic factors play an essential role in embryonic development (Herráez et al., 2017). However, further research is necessary to investigate the nature of this correlation and determine the exact role of sperm DNA damage on larval survival rate in barramundi. Additionally, it will be of interest to quantify the synergic effect of sperm DNA damage and oocyte repairing capacity on embryonic development as it has been done in other teleost species (Fernández-Díez et al., 2016; Fernández-Dıéz et al., 2018; Figueroa et al., 2020). This information would help refine threshold criteria for sperm quality and improve broodstock selection in hatcheries.

Finally, the lack of correlation of sperm DNA damage with the spawning outcome from the second spawning night indicates that sperm DNA damage might be associated with the presence of mature spermatozoa stored in the sperm duct and may not reflect the inherent quality of spermatogenesis of the individual. Therefore, it can be hypothesized that sperm DNA damage results from an intrinsic disruption post spermatogenesis (i.e., a cell apoptosis mechanism triggered in spermatozoa stored for an extended time in the sperm duct), rather than developmental or genetic disorders occurring during spermatogenesis. While sperm DNA damage in broodstock appeared to play a significant role in the

outcome and variability of mass-spawning events, additional factors, such as hormonal spawning stimulation, handling stress before spawning events (e.g., anaesthesia, gamete collection), social hierarchy, and tank breeding history, might disrupt broodstock's natural spawning behaviors. Consequently, even if strategies are found to synchronize spermiation and reduce levels of sperm DNA damage, it is unlikely that these will be sufficient to mitigate skewed parental contribution and facilitate the use of a mass-spawning breeding strategy for selective breeding programs. Furthermore, based on the findings of this research, visual assessment of milt characteristics and sperm motility performed before a spawning event to determine male reproductive condition might not assess the key parameters affecting fertilization success in barramundi, but only be useful to confirm the sex of the breeders. Consequently, the results of this study advocate for developing ARTs to gain control over the breeding process instead of investigating ways to manage skewed contributions using mass-spawning breeding strategies.

6.2 Future directions

Outcomes from this series of research studies focusing on aspects of barramundi fertility provide fundamental new knowledge on barramundi reproduction at both molecular and physiological levels. These studies also provide a standardized methodology to perform fertility assessment of male barramundi in future studies and hatcheries. The expertise gained with this project allows for the first time to question the reliability of accepted hatchery practices using a quantitative-based approach and to investigate breeding bottlenecks associated with current practices. In addition, the access to advanced sperm function assessments and a short-term sperm storage procedure for barramundi lays the foundation for developing ARTs, including cryopreservation and artificial fertilization to enhance breeding processes in the culture of the species. Implementing ARTs by the barramundi industry as the novel breeding method could transform the way the entire sector operates as it did for the Atlantic salmon industry. Namely, ARTs will permit increased control of the breeding process and seedstock production and potentially overcome the bottlenecks associated with mass-spawning. The development of ARTs will also open new horizons for research on barramundi by making genetic manipulation and

stem cell research more accessible. However, further research in male barramundi fertility is needed to reach these milestones. As such, four future research priorities have been identified to consolidate knowledge and methodologies based on the outcome of this research, including improvement of the sperm quality assessment method, sperm collection, sperm storage, and development of single-pairing procedures.

6.2.1 Improvement of sperm quality assessment method

The advanced sperm function assessments optimized in this project focus on important quality parameters. However, there are opportunities for improvement. Specifically, further studies should be performed focusing on developing an optimal artificial activation medium (AM) to improve the repeatability of the sperm motility assessment. It is known that the optimal ionic concentration of the AM is species-specific and that the osmolality and ionic composition of the AM affects sperm motility (Cosson et al., 2008b). In studies reported in this thesis, salt water from the recirculating system was collected and used as an AM to trigger sperm motility. Salt water was filtered using a 0.22 µm polyethersulfone (PES) syringe filter, and salinity and pH were adjusted to 30 ppt and pH 8.1, respectively. However, salt water has natural variations in ionic concentrations, such as Ca^{2+}, Mg^{2+}, and trace elements, and these differences could affect sperm motility. The development of an optimal artificial AM, following a similar experimental design to the one used in Chapter 3 for the optimization of the NAM, will enhance standardization and permit gaining a greater understanding of the intracellular motility mechanism barramundi spermatozoa.

Additional molecular assays could also provide critical information for further optimization of advanced procedures. In this study, the NAM was optimized for short-term chilled storage using viability and motility as key improvement measures. However, it was concluded that adding a mitochondrial integrity assay to the sperm quality assessment was required. Determining the mitochondrial integrity of barramundi spermatozoa will help identify if the cause of the low sperm

motility is related to the ATP content of the mitochondria, and thus, related to a lack of sperm maturity. This additional information could be crucial to further improving the chilled storage procedure and developing a reliable cryopreservation procedure to permit long-term storage and genetic banking of barramundi spermatozoa. Furthermore, as ATP acquisition by the mitochondria occurs during the final stage of sperm maturation (Schulz et al., 2010), knowing sperm ATP levels before and after hormonal stimulation could help optimize the hormonal treatment, including hormone type, dosage, and delivery methods and ultimately help identify the timing of peak sperm maturation and release and enable the collection of barramundi spermatozoa using the stripping method. In practical terms, the mitochondrial integrity assay could easily be integrated into the current standardized dual-staining Hoechst/Propidium iodide (PI) procedure of the sperm viability assay using Rhodamine 123 as an additional fluorescent dye to measure mitochondrial membrane potential (De Baulny et al., 1997; Zou et al., 2010). Alternatively, JC-1 fluorescent dye could be used to determine mitochondrial integrity by assessing sperm membrane potential ($\Delta\Psi M$; Figueroa et al., 2019). Finally, in addition to identifying the proportion of sperm mitochondrial integrity in sperm samples, determining the metabolic energy status of sperm samples before and after storage by measuring the ATP concentration could provide additional information for the optimization of procedures. Sperm ATP activity is commonly measured using luciferase bioluminescence assays and has been performed successfully in several teleost studies (Figueroa et al., 2019; Nguyen et al., 2016).

With the presence of high sperm DNA damage levels observed in spermatozoa from mature barramundi broodstock, further knowledge is required regarding barramundi sperm chromatin structure and which paternal genes are essential during early larval development. Specifically, it has been shown that during spermiogenesis, when spermatids undergo morphological changes and mature into spermatozoa, nucleic DNA is reorganized and condensed into a compact shape to improve genomic stability (Uribe et al., 2014; Ward, 2009). The packing organization of the DNA varies between species by either incorporating histone (-like) and/or protamine (-like) proteins in the matrix (Figueroa et al., 2020; González-Rojo et al., 2014; Ward, 2009). The different packing organizations affect how genes are compartmentalized in the chromatin and dictate how accessible genes are for transcription during early

larval development. As a result, genes readily available for transcription are also the most vulnerable to physical and chemical damage (Figueroa et al., 2020; Herráez et al., 2017). Characterizing barramundi sperm chromatin structure will provide valuable information into understanding sperm DNA damage in mature captive-bred barramundi. Furthermore, it is crucial to identify paternal genes susceptible to damage to find new genetic biomarkers and improve sperm quality diagnostics in barramundi. While the optimized TUNEL DNA fragmentation assay provides an overall genomic integrity status, the use of technologies, such as real-time PCR analysis and next-generation sequencing, can provide specific expression levels of candidate genes and identify genetic mutations induced by cryoinjuries, exposition to endocrine disrupters, and other factors generating free radicals (Figueroa et al., 2020; Herráez et al., 2017; Labbe et al., 2017). Several target candidate genes have already been identified in rainbow trout (*Oncorhynchus mykiss*), gilthead seabream (*Sparus aurata*), and zebrafish (*Danio rerio*) as biomarkers for genotoxic damage (e.g., *Hox*, *Sox2*, and *Eif1b*), and embryo quality (e.g., *Igf1* and *Gh*; Figueroa et al., 2020). Advanced genetic screening technologies for sperm and embryo quality assessment will permit measurable improvement of procedures ranging from the optimization of rearing practices to sperm handling and storage procedures for chilled storage and cryopreservation methodologies.

As spermatogenesis, sperm maturation, motility activation, and fertilization are dynamic processes, proteomic studies could provide an in-depth insight into the molecular mechanisms and potentially offer novel sperm quality-fertility biomarkers and avenues to improve storage procedures (Dietrich et al., 2017, 2016; Dietrich et al., 2014b, 2014a; Nynca et al., 2014). As shown in Chapter 4, adjusting the NAM formulation to mimic the ionic microenvironment of seminal plasma allowed significant improvement of the storage procedure. However, seminal plasma is not a simple ionic fluid; it is also composed of peptides/proteins, hormones, sugar, lipids, cytokines, and enzymes that have been shown to play an essential role in sperm protection and metabolism (Ciereszko et al., 2017; Figueroa et al., 2020). Investigating the seminal plasma/sperm proteome might help perfect NAM formulation and improve storage performance (Ciereszko et al., 2017; Figueroa et al., 2020). With the rapid growth of mass-spectrophotometry technology, proteomic characterization of seminal plasma in teleosts has certainly become more accessible (Ciereszko et al., 2017). However, initial groundworks will have to

be performed before conducting proteomic research as annotation of the transcriptome is limited for barramundi, as it is for several other teleost species (Ciereszko et al., 2017).

6.2.2 Enhancement of sperm collection

Collecting sperm samples reliably is the first critical procedure for implementing artificial fertilization procedures. While the optimization of the collection method of barramundi spermatozoa shown in Chapter 2 and 3 permits sperm samples to be obtained and maintained alive and motile over time, the volume of sperm collected remains insufficient to carry out the sperm quality assessment, store an aliquot for biobanking, and have an adequate volume to perform artificial fertilization. To address this issue, hormonal stimulation is commonly used in several aquaculture species to trigger hydration and artificially collect gametes using manual stripping (Migaud et al., 2013; Mylonas et al., 2010). In this research, as the collection of sperm samples was linked to the spawning schedule of the semi-commercial hatchery, the collection of sperm samples was only permitted before hormonal injection. Sperm samples were collected in these studies using a cannula rather than manual stripping because individuals rarely have milt that is sufficiently hydrated to allow successful stripping and avoid harming the broodstock. As such, only small volumes (i.e., 1–90 µl) of milt could be obtained from male broodstock. Consequently, it is critical to investigate the use of hormone manipulations to stimulate testicular hydration and final sperm maturation in male broodstock to enable manual stripping and the collection of sperm samples. To do so, it will be worthy of reconsidering the entire hormonal stimulation procedure in barramundi. The use of 25 $\mu g.kg^{-1}$ LHRHa hormone has been largely adopted by the barramundi industry for inducting broodstock to spawn based on Schipp et al. (2007). However, in earlier trials on the effect of hormone-induced spermatogenesis and spermiation in barramundi, Schipp (1993) concluded that

> "Based on these trials, it is not possible to develop a protocol, using hormone therapy that reliably enhances the breeding condition of male barramundi."

Therefore, there may be merit in reinvestigating the use of hormonal stimulation using sperm quality assessment to optimize the sperm collection procedure for the species. The optimization of the

procedure could be separated into three steps. Firstly, the effect of the different candidate hormones (i.e., LHRHa; purified human Chorionic Gonadotropin, hCG; synthetic agonists of gonadotropin-releasing hormone, GnRHa; 17β-estradiol, E_2; 17,20β-dihydroxypregn-4-en-3-one, 17,20β-P) should be re-examined. Secondly, the effect of the hormone delivery (i.e., slow-release intra-muscular implant and repeated injection) should also be pursued. Finally, the best time window to collect sperm samples post-hormonal stimulation should be determined. Following these three steps, the resulting sperm collection procedure has the potential to improve the volume of sperm collected from micro to millimeters and achieve volumes as reported from wild-caught barramundi (Leung, 1987; Palmer et al., 1993). At the same time, a similar approach is required to facilitate the collection of eggs by using manual stripping to ensure mature eggs are collected and/or could be artificially matured *in vitro*, thereby achieving fertilization in the presence of spermatozoa.

6.2.3 Enhancement of sperm storage

To improve sperm storage methodologies, additional factors such as the dilution factor with NAM and salt water could be further investigated. In this thesis, a 1:20 (sperm/NAM) and 1:50 (diluted sperm/saltwater) ratio was used, resulting in a final dilution of 1:1000. While the final 1:1000 dilution was identified in Chapter 3 to provide the most accurate sperm concentration assessments using CASA, the effect of the dilution ratios through the two-step dilution procedure has not been examined regarding sperm motility. It has been shown in several studies that specific dilution ratios of NAM and salt water can affect sperm motility and storage outcomes (Fauvel et al., 1999; Jing et al., 2009; Peñaranda et al., 2010; Tan-Fermin et al., 1999).

In addition, sperm storage performance could be improved by supplementing the NAM with additives. Additives such as bovine serum albumin (BSA), fetal bovine serum (FBS), and egg yolk are frequently used in NAM to improve sperm membrane integrity and motility in chilled storage (Fauvel et al., 1999; Gallego et al., 2013b; Kowalski et al., 2014; Peñaranda et al., 2010). Analyzing the effect of these additives on the performance of chilled storage of barramundi spermatozoa would be a prerequisite

before further modifications to the NAM. Moreover, gaining a better understanding of the effect of these supplements on chilled storage will also help in adjusting the formulation of the NAM for the future cryopreservation procedure.

Ultimately, the knowledge on reproduction and sperm biology gained in developing the chilled storage procedure will provide a robust foundation for developing cryopreservation procedures. While chilled sperm storage will facilitate the development of an artificial fertilization technology, cryopreservation will ultimately allow the long-term storage of sperm samples. This means that even though male broodstock change sex and become female and/or are no longer in breeding conditions, their milt will be stored at -196 °C in liquid nitrogen and will be available for use at any time for artificial fertilization. Sperm cryopreservation of wild-caught barramundi has been achieved in the past with the addition of dimethylsulphoxide (DMSO) at a concentration of 5% to the NAM (Leung, 1987; Palmer et al., 1993). However, the industry has not adopted this process, possibly due to a need to optimize the methodology. Therefore, it is warranted to test and optimize the current cryopreservation methodology using barramundi sperm quality assessment to render cryopreservation technology accessible to the entire barramundi aquaculture industry.

6.2.4 Achieve control over male-female pairing

Breeding barramundi using the mass-spawning breeding method has been shown to generate highly variable outcomes, potentially affecting the outcomes of selective breeding programs (Domingos et al., 2014, 2013; Frost et al., 2006; Gjedrem, 2010; Loughnan et al., 2013). Therefore, the requirement for new breeding technology to control spawning for barramundi is irrefutable. As such, to gain control over spawning outcomes, two possible avenues have been identified, including the development of a single-pair breeding method for barramundi and artificial fertilization. The development of a single-pair breeding method for barramundi, if successful, could be a short-term solution that would immediately permit the implementation of selective breeding programs by bringing increased control over pairings and broodstock contribution to the subsequent generation of progeny. Although the idea

of breeding species with strict natural mass-spawning behavior in a one-on-one mating design seems counterintuitive and often considered impossible by hatchery operators, to date, there is no report of any attempt to breed barramundi using this mating design. With an optimized hormonal treatment and tank design, it is possible that the fertilization of eggs by male broodstock could be achieved despite the lack of social cues. However, artificial fertilization is the technology required in the long term for controlling breeding effectively and increasing the benefits-cost ratio of selective breeding programs for barramundi (Macbeth and Palmer, 2011; Robinson et al., 2010).

6.3 Conclusion

Bottlenecks associated with breeding barramundi in captivity using the mass-spawning breeding strategy remain to be overcome. However, the findings identified in this thesis provide fundamental knowledge and procedures to help resolve these bottlenecks. A range of optimized sperm quality assays and procedures to diagnose, collect, and reliably store sperm samples are available for the first time. As well, new insights into the relationship between male broodstock spawning performances and their fertility in captivity have been shown. The data generated in this research paves the way for future studies on fertility and the development of ARTs, which will play a pivotal role in driving transformative innovation in the Australian barramundi aquaculture industry over the next decade.

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Appendices

Appendix A Sperm quality assessments using published methodologies on barramundi (*Lates calcarifer*) broodstock induced sperm lysis: a pilot study

Background:

Visual sperm quality assessments of barramundi broodstock are performed in most Australian hatcheries to verify the sex and the maturation stage before inducing a spawning event. As per Australian barramundi industry standard practice, cannulation of the gonads is performed on anaesthetized broodstock, and an aliquot of milt is diluted with salt water to trigger motility activation. Specifically, a drop of raw milt is placed on a glass slide immediately after collection, and the sample is assessed under a bright-field stereomicroscope using an X 10 objective. Once the presence of spermatozoa is confirmed, the milt on the glass slide is mixed with a drop of saltwater collected from the tank, and a visual estimate of total motility is performed. Using this method, barramundi sperm total motility usually ranged from 80 to 95%, confirming spermatozoa are viable and mature. While this method provides a quick understanding of the overall condition of the broodstock, the development of a reliable and quantitative assessment of sperm quality in barramundi was required to investigate barramundi reproductive biology, and more specifically for this research project, the origin of inconsistencies in paternal contribution during mass-spawning events in hatcheries. Therefore, a pilot study was conducted to test standard sperm assessment methods used in livestock and advanced commercial fish species (e.g., Atlantic salmon), including sperm morphology, sperm motility, sperm count, and sperm viability to gather general characteristics of barramundi spermatozoa.

Methods

Animals

Barramundi (*Lates calcarifer*) were maintained at the Marine and Aquaculture Research Facility (MARF), James Cook University, Townsville, Australia. Fish were mature adults and maintained in breeding conditions in 28,000 L tanks held in standard maturation conditions (i.e., 30 °C, 30 ppt salinity,

and 16 h light: 8 h dark cycle). Fish were fed a formulated maturation diet 4 days per week at 1% body weight per feed (LANSY-Breed M, INVE Aquaculture). Pilot experiments were approved by the James Cook University Animal Ethics Committee (A2406).

Sperm collection and processing

Milt was collected from males in spawning conditions. Selected broodstock were sedated in an anaesthetic bath containing iso-eugenol at 40 mg/L (AQUI-S®, New Zealand) prior to sperm collection (Schipp et al., 2007). The gonopore of males was rinsed with distilled water and dried to prevent saltwater prematurely activating spermatozoa during collection. Milt samples were obtained by cannulation with a 1.72 mm diameter cannula (PE-90 polyethylene tubing, Becton Dickinson, Sparks, MD, USA), and an aliquot visually assessed by bright field microscopy for quality control (presence of blood, urine contamination, premature sperm activation, or failure to activate on contact with saltwater). Milt volume (V) was calculated by measuring the length of raw sperm in the cannula according to:

$$V = \pi * r^2 * h$$

Where *r* is the radius of the cannula (*r* = 0.43 mm) and *h* is the sample length measured in millimeters. Manual counts were performed using an improved Neubauer haemocytometer. The manual calculation of sperm concentration was determined using the mean number of sperm per 5 small squares across both chambers (*N*), multiplied by the square multiplication factor 50,000 for (R), and multiplied by the dilution factor.

$$[c] = N \times 50{,}000 \times dilution\ factor$$

The non-activating medium used to dilute sperm samples was a modified Marine Ringer's solution (MRS) consisting of 124.1 mM NaCl, 5.1 mM KCl, 1.6 mM CaCl2•2H2O, 1.1 mM MgSO4•7H2O, 0.1 mM NaHCO3, 2.6 mM NaH2PO4•2H2O, and 5.6 mM D^+ glucose, pH 7.4 and 260 mOsm (based on Palmer, 1993). MRS was selected as the non-activating medium as it was previously used by Palmer (1993) to collect milt from wild barramundi and as a medium for a cryopreservation trial. Sperm motility was activated using saltwater consisting of 404.9 mM Na^+, 459.1 mM Cl^-, 10.1 mM K^+, 9.7 mM Ca^{2+}, 0.1 mM PO_4^{-3}, pH 8.1 and 900 mOsm.

Reports

Trial 1: Testing standard sperm quality assessment procedures, including sperm concentration, sperm motility, and morphology, to collect preliminary measurements of barramundi sperm characteristics.

In this first trial, sperm samples from broodstock (n = 4) were successfully collected. Milt volume was directly determined post-collection. Milt was transferred into a 1.5 mL Eppendorf tube. A 2 μL aliquot of raw milt was then diluted into 498 μL MRS and held in ice. A second 4 μL aliquot of diluted milt was collected and diluted with 16 μL of saltwater to activate sperm motility before being loaded into a Leja slide chamber (Leja Products B. V., Nieuw Vennep, Amsterdam, The Netherlands). The final dilution used was 1:1250. This process was repeated three times to account for technical replicate. As the CASA software (i.e., AndroVision®, version 1.1 software, Minitüb GmbH, Tiefenbach, Germany) was unable to detect barramundi spermatozoa using standard detection profiles, motility duration was used as a measure of sperm motility. Once the sperm motility assessment was completed (within 30 min of collection), sperm concentration was determined using an improved Neubauer haemocytometer.

Overall, milt samples collected ranged from 7 to 39 μL with an average sperm concentration of 28.5 ± 4.4 x 10^9 sperm/L and a total sperm motility duration of 2 min 27 s (Table 1).

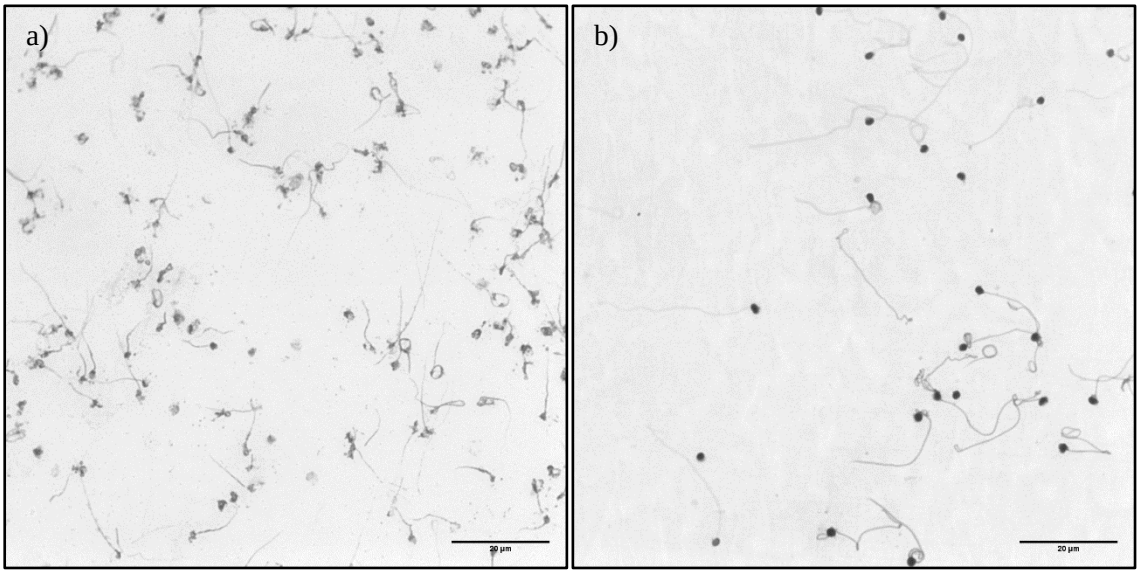
Table 1: Barramundi (*Lates calcarifer; n* = 4) milt characteristics, including milt volume (μL), sperm concentration (x 10^9 sperm/mL) and sperm motility duration (s). Data are displayed as mean ± standard error.

Fish ID	**Milt volume (μL)**	**Manual sperm concentration (x 10^9 sperm/mL)**	**Motility duration until immobility (min:s)**
F1	39	37.0	3:26 ± 1:27
F2	19	26.6	1:28 ± 0:07
F3	29	24.6	3:15 ± 0:57
F4	7	25.9	1:38 ± 0:42
Average	23.5 ± 10.6	28.5 ± 4.4 x 10^9	2:27 ± 0:54

It was noted that sperm motility was performed within ~2 min of collection and that spermatozoa were looking healthy (i.e., round head with clear boundaries with straight and long flagella). However, at 30 min post collection, spermatozoa diluted in MRS and held in ice were used for manual sperm concentration. It was observed that spermatozoa exhibited cellular damages, including swollen heads

and coiled and bent flagella. At 1 h post-collection, spermatozoa diluted in MRS and held in ice were reassessed for sperm motility and failed to activate in contact with salt water (i.e., immotile or slight head wobble without motion). At three hours post collection, spermatozoa diluted in MRS and held in ice were used for morphology assessment using SpermBlue® (Microptic, SL, Barcelona, Spain) fixative and stain solution as per manufacturer instruction. However, while spermatozoa could be identified on the slide were disintegrated (Figure 1a). The staining procedure was repeated; however, spermatozoa were incubated for 15 mins in PFA before being smeared on the glass slide and stained. Using this method, spermatozoa retained the stain and were identifiable (Figure 1b). Spermatozoa had swollen heads as well as coiled and broken flagella, as observed under the microscope when performing the manual sperm concentration assessment (Figure 1b). Similar cellular damages were observed in spermatozoa exposed to hypotonic media, such as in the Northern pike (*Esox Lucius* L.; Alavi et al., 2009) and tilapia (*Oreochromis mossambicus*; Morita et al., 2004). It was, therefore, hypothesized that barramundi spermatozoa exposed to MRS were subject to a hypotonic shock causing heads to swell and flagella to coil or break.

Figure 1: Barramundi (*Lates calcarifer*) spermatozoa stained with SpermBlue after 1 h incubation in Marine Ringer's solution in ice (a) without pre-PFA fixation (magnification X 50) and (b) with 15 min PFA fixation prior staining (magnification X 50).



Trial 2: Investigation of the causes of cellular damages in barramundi spermatozoa exposed to the non-activating medium: Marine Ringer's solution.

In this second trial, male barramundi broodstock (n = 4) were assessed for sperm cannulation, and three individuals provided a milt sample. Milt volume and sperm concentration were within the range of what was previously reported in trial 1 (Table 2). However, the duration of sperm motility post-activation was extremely brief for all milt samples collected (i.e., < 15 s; Table 2).

Table 2: Barramundi (*Lates calcarifer; n* = 4) milt characteristics, including milt volume (μL), sperm concentration (x 10^9 sperm/mL) and sperm motility duration (s). Data are displayed as mean ± standard error.

Fish ID	**Milt volume (μL)**	**Manual sperm concentration (x 10^9 sperm/mL)**	**Motility duration until immobility**
F1	36.1	37.6	< 15 s
F2	20.5	24.7	< 15 s
F3	22.8	26.6	< 15 s
F4	NA	NA	NA
Average	26.5 ± 6.0	29.6 ± 4.9	2:27 ± 0:54

An osmolality assessment of the different solutions used for sperm concentration (i.e., MRS), sperm motility (i.e., MRS and salt water), sperm morphology (i.e., PFA in MRS), and sperm viability (i.e., EDTA in PBS) assessments was performed as well as fresh pooled milt samples using a freezing point depression osmometer (Osmomat 030; Genotec GmbH, Berlin, Germany). The osmolality of barramundi milt was 403 mOsm/kg, whereas MRS was 261 mOsm/kg. This preliminary data supported the hypothesis that the MRS was not isotonic to barramundi milt.

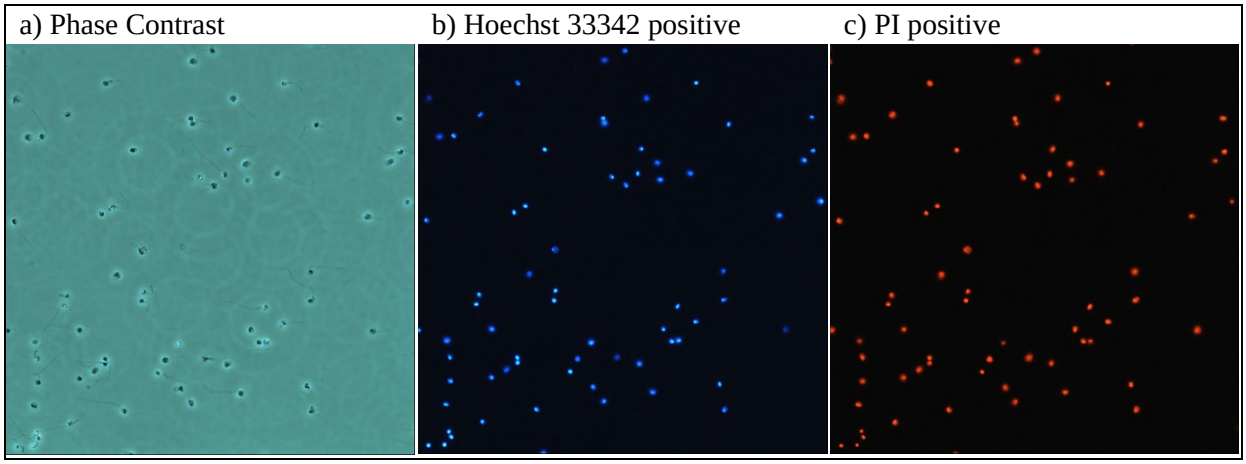
Table 3: Osmolality measurements of barramundi milt and solutions used to perform sperm quality assessment. MRS: Marine Ringer's solution (diluent medium used for sperm concentration and motility assessment. PBS and EDTA in PBS: Phosphate-buffered saline (diluent medium used for sperm viability). PFA in MRS: Paraformaldehyde (fixative used for sperm morphology).

	Pooled milt samples (~30 μL)	**MRS**	**Filtered PBS**	**EDTA in PBS**	**PFA in MRS**	**Filtered saltwater**
Osmolality (mOsm/kg)	406 ± 1.9	261	290	288	1595	1006

Following this assessment, sperm viability assay was performed and determined using a dual fluorescent staining method (i.e., Hoechst 33342/Propidium Iodide; PI). Hoechst 33342 is a cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA and is used to

identify spermatozoa from debris. Propidium iodide (PI) is a cell-impermeant nuclear counterstain that emits red fluorescence and is used to detect dead cells in a population. Samples were analyzed using fluorescent microscopy. Micrographs were captured using an Olympus BX53/CoolLED pE-300W fluorescent microscope at 50 X final magnification (i.e., 0.5 X C-Mount adaptor coupled with a 100 X objective). After 1 h incubation in ice, barramundi spermatozoa held in MRS showed swollen heads, coils, folding, and blebs in flagella (Figure 2a). Sperm viability staining also showed that spermatozoa had damaged cellular plasma membrane and therefore considered dead (Figure 2b and c).

Figure 2: Barramundi spermatozoa (a) under phase-contrast microscopy and stained for viability using (b) Hoechst 33342, and (c) propidium iodide



Conclusion

Based on data collected in trials 1 and 2, milt samples collected from barramundi broodstock using cannulation were about 25 μL. The limited amount of milt retrieved from the barramundi broodstock made sperm handling difficult and the requirement for a non-activation medium a priority to ensure sufficient milt can be aliquoted and are available for the different sperm quality assessments. While there are no quantitative records of milt volume of barramundi broodstock available, Palmer (1993) noted in his study that he collected milt samples of several millimeters from wild barramundi using strip spawning. Despite different milt collection methods used, it can be inferred that barramundi broodstock assessed were in the early stage of spermiation and were not entirely in spawning conditions at the time of the cannulation. Furthermore, before being activated with saltwater, barramundi spermatozoa were healthy and motile after a short incubation (~30 s) in MRS. However, incubation of

spermatozoa < 30 min in MRS was detrimental to the integrity of barramundi spermatozoa and showed similar cellular damages than spermatozoa exposed to a hypo-osmotic shock (Figure 1 and 2a; Alavi et al., 2009; Morita et al., 2004). Sperm viability assessment indicated that all spermatozoa were dead after 1 h incubation in MRS. Finally, the osmolality assessment of barramundi milt and MRS confirmed that MRS induced a hypo-osmotic shock to barramundi spermatozoa, resulting in cellular damages and dead. Therefore, it is recommended to investigate further barramundi spermatozoa physiological requirements and adjust the composition of the non-activating medium accordingly.

Appendix B Sperm motility characteristics for each male barramundi *(Lates calcarifer*).

Male	TM (%)	PM (%)	Slow (%)	Medium (%)	Fast (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)	BCF (Hz)
1	15.8 ± 5.6	10.9 ± 3.7	4.8 ± 2.2	7.8 ± 2.8	3.1 ± 1.9	68.6 ± 7.5	44.1 ± 7.1	58.3 ± 8.0	53.9 ± 4.5	70.5 ± 3.9	72.8 ± 3.9	0.49 ± 0.02	11.1 ± 1.0	11.1 ± 1.0
2	13.4 ± 9.4	7.3 ± 6.0	6.1 ± 3.4	6.9 ± 6.2	0.3 ± 0.3	43.9 ± 3.5	18.9 ± 2.3	28.4 ± 3.7	38.1 ± 3.4	61.1 ± 3.4	57.3 ± 3.1	0.51 ± 0.02	11.2 ± 1.1	11.2 ± 1.1
3	17.8 ± 6.9	14.0 ± 6.3	3.7 ± 2.0	7.5 ± 3.6	6.5 ± 3.6	98.6 ± 12.9	70.7 ± 12.9	86.8 ± 14.1	54.8 ± 5.9	69.6 ± 4.9	70.9 ± 5.4	0.52 ± 0.03	14.0 ± 1.9	14.0 ± 1.9
4	11.3 ± 1.3	5.7 ± 1.5	5.6 ± 0.9	4.9 ± 1.1	0.8 ± 0.5	46.3 ± 4.6	37.4 ± 4.8	40.9 ± 4.9	68.2 ± 4.4	81.0 ± 3.3	78.8 ± 3.4	0.39 ± 0.01	11.4 ± 1.1	11.4 ± 1.1
5	31.8 ± 5.6	8.7 ± 1.6	23.1 ± 5.0	6.3 ± 1.5	2.2 ± 0.9	40.4 ± 3.2	23.8 ± 2.8	29.5 ± 3.2	45.2 ± 1.9	72.1 ± 1.7	58.0 ± 1.6	0.44 ± 0.01	6.1 ± 0.4	6.1 ± 0.4
6	26.4 ± 8.0	1.7 ± 0.9	24.7 ± 7.2	1.7 ± 0.9	0.0 ± 0.0	20.8 ± 1.5	8.3 ± 1.9	10.6 ± 1.8	34.0 ± 4.4	67.2 ± 3.6	46.5 ± 3.8	0.33 ± 0.01	4.0 ± 0.8	4.0 ± 0.8
7	27.6 ± 3.3	7.0 ± 1.9	20.6 ± 1.4	2.7 ± 0.5	4.3 ± 2.4	60.9 ± 14.5	40.1 ± 13.0	49.3 ± 14.4	46.5 ± 5.2	73.3 ± 5.3	59.3 ± 4.1	0.54 ± 0.07	4.4 ± 0.7	4.4 ± 0.7
8	52.4 ± 1.9	20.3 ± 5.0	32.1 ± 4.1	7.9 ± 2.6	12.3 ± 2.5	65.0 ± 6.7	50.3 ± 6.5	54.0 ± 6.7	57.7 ± 2.5	84.0 ± 1.8	65.3 ± 2.2	0.47 ± 0.02	6.2 ± 0.6	6.2 ± 0.6
Mean	24.5 ± 4.4	9.4 ± 1.9	15.1 ± 3.7	5.7 ± 0.8	3.7 ± 1.5	55.6 ± 8.2	36.7 ± 6.9	44.7 ± 8.2	49.8 ± 3.9	72.3 ± 2.6	63.6 ± 3.7	0.46 ± 0.02	8.6 ± 1.3	8.6 ± 1.3

Data are displayed as mean ± standard error. Total motility (TM; VCL ≥ 15 μm/s); Progressive motility (PM; 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); curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), straightness of the average path (STR), linearity of the curvilinear path (LIN), wobble (WOB) and beat-cross frequency (BCF).

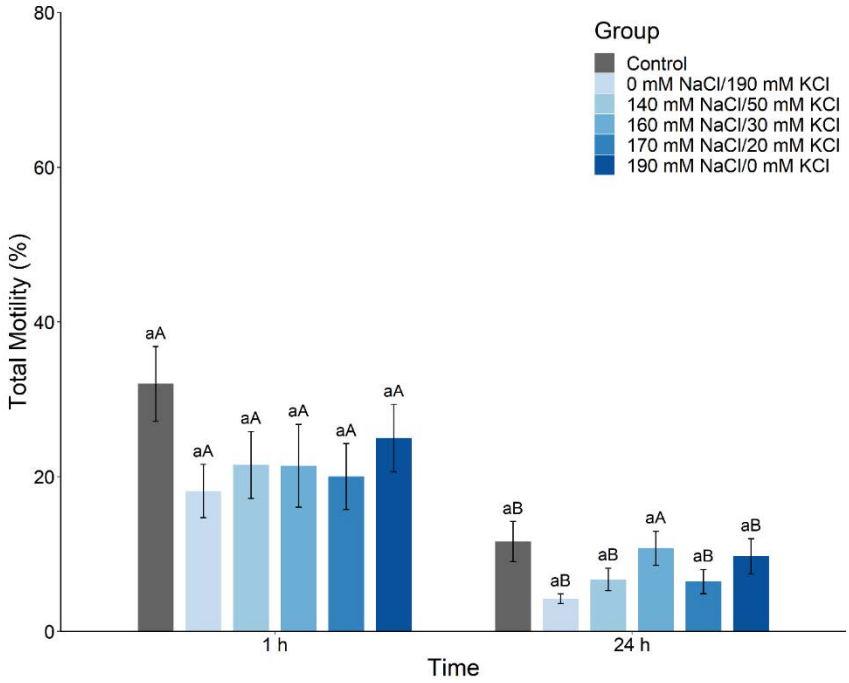
Appendix C Effect of $NaHCO_3$ buffered NAM Na^+ and K^+ concentration on sperm motility.

Methods:

After determining the optimal NAM osmolality, the ratio of NaCl and KCl included in the medium was assessed to determine whether a Na^+/K^+ ion ratio similar to barramundi seminal plasma will improve the motility of spermatozoa after activation with saltwater. Different $NaHCO_3$ buffered NAM treatments were prepared with the following NaCl/KCl concentrations: 0 mM NaCl/190 mM KCl, 140 mM NaCl/50 mM KCl, 160 mM NaCl/30 mM KCl, 170 mM NaCl/20 mM KCl, 190 mM NaCl/0 mM KCl. All solutions maintained an osmolality of 400 mOsm/kg and were adjusted to pH 7.4 with 0.1 M NaOH (for all other treatment groups). Sperm samples from ten males were undiluted (milt control) or diluted 1:10 in the different NAMs and incubated at 4 ℃ for 1 h and 24 h before motility was assessed by CASA.

Results:

After 1 h incubation, motility of barramundi spermatozoa did not differ across the wide range of Na^+ and K^+ ion concentrations nor milt control (Figure 1; Table 1). Overall motility was low across all media, with the highest motility observed in the milt control. After 24 h incubation, sperm motility significantly declined for all treatments except 160 mM NaCl/30 mM KCl (Figure 1), with the highest motility again observed in the milt control. Moreover, spermatozoa incubated in the 0 mM NaCl/190 mM KCl showed significantly lower progressive, medium, and fast motility than the undiluted milt control. Similarly, significantly lower swimming speeds and straight trajectories were observed (VCL, VSL, VAP, LIN, WOB, and BCF; Table 1). However, except for the 0 mM/190 mM KCl medium, the motility and trajectory of spermatozoa did not differ between different Na^+ and K^+ treatments (Table 1).



Appendix B Figure 1 Total motility of barramundi (*Lates calcarifer*) spermatozoa (n = 10) incubated for 1 h and 24 h at 4 °C undiluted (milt control) or diluted in $NaHCO_3$ buffered buffered non-activating medium (NAM: 0-190 mM NaCl, 0-190 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $NaHCO_3$, 2.6 mM NaH_2PO_4. $2H_2O$ and 5.6 mM D^+ glucose at pH 7.4 and 400 mOsm/kg) containing different NaCl/KCl concentrations. Data are displayed as mean ± SEM. Different lowercase letters indicate a significant difference between concentration treatments within an incubation period, and different capital letters indicate a significant difference between incubation periods at the same concentration treatment ($P < 0.05$).

Appendix B Table 1 Motility parameters of barramundi, *Lates calcarifer*, (n = 10) testicular spermatozoa incubated for 1 h and 24 h undiluted (milt control) or diluted in $NaHCO_3$ buffered non-activating medium (NAM: 0-190 mM NaCl, 0-190 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $NaHCO_3$, 2.6 mM NaH_2PO_4. $2H_2O$ and 5.6 mM D^+ glucose at pH 7.4 and 400 mOsm/kg) containing different NaCl/KCl concentrations.

	1 h incubation						24 h incubation					
Parameter	**Control**	**0 mM NaCl/ 190 mM KCl**	**140 mM NaCl/ 50 mM KCl**	**160 mM NaCl/ 30 mM KCl**	**170 mM NaCl/ 20 mM KCl**	**190 mM NaCl/ 0 mM KCl**	**Control**	**0 mM NaCl/ 190 mM KCl**	**140 mM NaCl/ 50 mM KCl**	**160 mM NaCl/ 30 mM KCl**	**170 mM NaCl/ 20 mM KCl**	**190 mM NaCl/ 0 mM KCl**
TM (%)	32.0 ± 4.8^{aA}	18.1 ± 3.5^{aA}	21.5 ± 4.3^{aA}	21.4 ± 5.4^{aA}	20.0 ± 4.3^{aA}	25.0 ± 4.4^{aA}	11.6 ± 2.6^{aB}	4.2 ± 0.6^{aB}	6.7 ± 1.5^{aB}	10.8 ± 2.2^{aA}	6.5 ± 1.6^{aB}	9.7 ± 2.3^{aB}
PM (%)	18.5 ± 3.2^{aA}	8.1 ± 2.1^{aA}	10.2 ± 2.9^{aA}	10.7 ± 3.0^{aA}	8.4 ± 2.2^{aA}	12.1 ± 2.9^{aA}	5.4 ± 1.8^{aB}	0.5 ± 0.2^{bB}	2.0 ± 0.8^{abB}	3.5 ± 1.1^{abB}	1.8 ± 0.8^{abB}	3.1 ± 1.0^{abB}
Slow (%)	13.5 ± 2.3^{aA}	10.1 ± 1.8^{aA}	11.3 ± 1.9^{aA}	10.7 ± 2.3^{aA}	11.6 ± 2.5^{aA}	12.9 ± 1.7^{aA}	6.2 ± 1.1^{aB}	3.7 ± 0.5^{aB}	4.7 ± 0.8^{aB}	7.3 ± 1.3^{aA}	4.6 ± 0.8^{aB}	6.6 ± 1.4^{aB}
Medium (%)	11.6 ± 1.6^{aA}	5.1 ± 1.3^{bA}	6.1 ± 1.7^{abA}	6.6 ± 1.6^{abA}	5.5 ± 1.4^{abA}	8.4 ± 1.9^{abA}	3.9 ± 1.3^{aB}	0.4 ± 0.1^{bB}	1.3 ± 0.6^{abB}	2.7 ± 0.8^{abB}	1.4 ± 0.6^{abB}	2.5 ± 0.9^{abB}
Fast (%)	6.8 ± 1.9^{aA}	3.0 ± 1.1^{aA}	4.2 ± 1.5^{aA}	4.1 ± 1.9^{aA}	2.9 ± 1.0^{aA}	3.7 ± 1.2^{aA}	1.5 ± 0.7^{aB}	0.1 ± 0.1^{bB}	0.7 ± 0.3^{abB}	0.8 ± 0.4^{abB}	0.4 ± 0.2^{abB}	0.7 ± 0.3^{aB}
VCL (µm/s)	63.8 ± 6.7^{aA}	48.2 ± 8.3^{aA}	54.3 ± 10.8^{aA}	51.5 ± 8.3^{aA}	49.4 ± 9.5^{aA}	52.8 ± 6.4^{aA}	42.9 ± 6.4^{aB}	22.7 ± 1.6^{bB}	32.3 ± 3.9^{abB}	33.8 ± 4.1^{aB}	29.6 ± 3.4^{abB}	38.1 ± 5.1^{aB}
VSL (µm/s)	39.2 ± 5.5^{aA}	29.0 ± 7.8^{aA}	33.6 ± 10.4^{aA}	29.4 ± 6.9^{aA}	28.3 ± 8.6^{aA}	27.5 ± 4.9^{aA}	20.0 ± 4.9^{aB}	3.1 ± 1.0^{bB}	10.4 ± 3.3^{aB}	13.1 ± 4.4^{aB}	8.9 ± 2.9^{abB}	12.2 ± 3.1^{aA}
VAP (µm/s)	52.3 ± 7.1^{aA}	34.7 ± 8.6^{aA}	41.9 ± 11.7^{aA}	39.5 ± 8.8^{aA}	36.5 ± 10.0^{aA}	39.8 ± 6.9^{aA}	30.8 ± 7.0^{aB}	7.3 ± 1.6^{bB}	17.3 ± 4.5^{abB}	19.0 ± 4.8^{abB}	14.6 ± 3.8^{abB}	21.9 ± 4.9^{aB}
LIN (%)	48.8 ± 2.8^{aA}	36.5 ± 4.7^{aA}	39.8 ± 3.9^{aA}	41.5 ± 4.3^{aA}	36.1 ± 4.2^{aA}	41.1 ± 3.4^{aA}	29.9 ± 5^{aB}	11.8 ± 1.9^{bB}	19.1 ± 3.9^{abB}	25.5 ± 5.2^{aB}	19 ± 4.7^{abB}	21.6 ± 4.1^{abB}
STR (%)	66.6 ± 2.6^{aA}	59.9 ± 3.3^{aA}	62.2 ± 2.5^{aA}	62.4 ± 3.7^{aA}	57.8 ± 2.7^{aA}	61.7 ± 2.8^{aA}	49 ± 3.8^{abB}	34.8 ± 3.2^{bB}	40.4 ± 3.8^{abB}	52.9 ± 4.1^{abB}	42.2 ± 4.9^{abB}	44.3 ± 4.2^{abB}
WOB (%)	66.2 ± 2.4^{aA}	49.7 ± 4.4^{bA}	54.6 ± 4.2^{bA}	58.3 ± 4^{abA}	52.2 ± 4.3^{bA}	58.7 ± 3.9^{abA}	50.7 ± 6^{aB}	28.4 ± 2^{bB}	36.8 ± 4.3^{abB}	40.8 ± 5.1^{abB}	36.3 ± 4.8^{abB}	41.7 ± 4.1^{abB}
ALH (µm)	0.47 ± 0.01^{aA}	0.44 ± 0.02^{aA}	0.44 ± 0.01^{aA}	0.47 ± 0.01^{aA}	0.45 ± 0.01^{aA}	0.47 ± 0.01^{aA}	0.42 ± 0.03^{aB}	0.40 ± 0.02^{aA}	0.41 ± 0.01^{aA}	0.43 ± 0.01^{aB}	0.41 ± 0.01^{aB}	0.46 ± 0.03^{aA}
BCF [Hz]	11.3 ± 0.8^{aA}	8.2 ± 1.1^{bA}	8.8 ± 0.9^{abA}	8.8 ± 1.0^{abA}	7.9 ± 0.8^{bA}	9.7 ± 0.8^{abA}	7.2 ± 1.2^{aB}	3.3 ± 0.6^{aB}	5.6 ± 1.1^{aB}	6.3 ± 1.0^{aB}	5.4 ± 1.2^{aA}	6.5 ± 0.9^{aB}

Data are displayed as mean ± standard error. Total motility (TM; VCL ≥ 15 µm/s); Progressive motility (PM; 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; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency. Different lowercase letters indicate a significant difference between concentration treatments within an incubation period, and different capital letters indicate a significant difference between incubation periods at the same concentration treatment ($P < 0.05$).

Appendix D Motility parameters of barramundi, *Lates calcarifer*, (n = 10) testicular spermatozoa incubated at 4 °C for 1 h and 24 h undiluted (milt control) or diluted in $NaHCO_3$ buffered non-activating medium (NAM: 182.4 mM NaCl, 5.1 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $NaHCO_3$, 2.6 mM $NaH_2PO_4 \cdot 2H_2O$, 5.6 mM D^+ glucose) with different pH.

	1 h incubation						24 h incubation					
Parameter	**Control**	**pH 6.5**	**pH 7.4**	**pH 7.8**	**pH 8.1**	**pH 8.5**	**Control**	**pH 6.5**	**pH 7.4**	**pH 7.8**	**pH 8.1**	**pH 8.5**
TM (%)	41.8 ± 3.8^{aA}	33.4 ± 3.0^{abA}	23.2 ± 2.0^{bcA}	24.2 ± 2.8^{bcA}	17.4 ± 2.8^{cA}	17.2 ± 3.3^{cA}	17.2 ± 2.1^{aB}	11.2 ± 2.4^{aB}	1.8 ± 0.5^{bB}	1.3 ± 0.2^{bB}	1.8 ± 0.4^{bB}	1.3 ± 0.2^{bB}
PM (%)	26.8 ± 3.3^{aA}	21.7 ± 2.5^{abA}	13.9 ± 1.5^{bcA}	15.7 ± 2.1^{abcA}	10.9 ± 2.1^{cA}	10.4 ± 2.5^{cA}	8.8 ± 1.4^{aB}	6.1 ± 1.5^{aB}	0.2 ± 0.2^{bB}	0.1 ± 0.0^{bB}	0.1 ± 0.1^{bB}	0.2 ± 0.1^{bB}
Slow (%)	14.9 ± 1.6^{aA}	11.8 ± 1.0^{abA}	9.3 ± 0.9^{bcA}	8.5 ± 0.8^{bcA}	6.5 ± 0.8^{cA}	6.8 ± 1.0^{cA}	8.4 ± 1.0^{aB}	5.1 ± 1.0^{bB}	1.6 ± 0.3^{cB}	1.2 ± 0.2^{cB}	1.7 ± 0.4^{cB}	1.1 ± 0.2^{cB}
Medium (%)	12.5 ± 1.4^{aA}	7.6 ± 0.9^{abA}	5.7 ± 0.8^{bA}	5.5 ± 0.5^{bA}	4.2 ± 0.8^{bA}	4.4 ± 1.0^{bA}	3.8 ± 0.6^{aB}	3.5 ± 0.9^{aB}	0.2 ± 0.1^{bB}	0.1 ± 0.0^{bB}	0.1 ± 0.1^{bB}	0.2 ± 0.1^{bB}
Fast (%)	14.3 ± 3.1^{aA}	14.0 ± 2.3^{aA}	8.1 ± 1.1^{abA}	10.2 ± 1.9^{abA}	6.7 ± 1.4^{abA}	5.9 ± 1.6^{bA}	4.9 ± 0.9^{aB}	2.6 ± 1.0^{bB}	0.1 ± 0.1^{cB}	0.0 ± 0.0^{cB}	0.0 ± 0.0^{cB}	0.0 ± 0.0^{cB}
VCL (µm/s)	89.3 ± 9.4^{aA}	111.3 ± 11.7^{aA}	95.9 ± 7.2^{aA}	101.1 ± 7.0^{aA}	95.0 ± 9.3^{aA}	88.0 ± 9.5^{aA}	77.9 ± 7.2^{aA}	65.1 ± 10.9^{aB}	22.2 ± 1.8^{bB}	21.2 ± 1.1^{bB}	20.7 ± 1.0^{bB}	20.2 ± 1.1^{bB}
VSL (µm/s)	37.6 ± 5.4^{aA}	52.7 ± 6.7^{aA}	52.9 ± 7.2^{aA}	49.4 ± 4.7^{aA}	51.9 ± 8.0^{aA}	46.9 ± 8.6^{aA}	40.1 ± 7.1^{aA}	24.4 ± 9.2^{aB}	3.7 ± 0.9^{bB}	3.1 ± 0.3^{bB}	2.1 ± 0.3^{cB}	2.6 ± 0.2^{bcB}
VAP (µm/s)	76.2 ± 9.5^{aA}	101.1 ± 11.3^{aA}	87.2 ± 7.1^{aA}	91.7 ± 7.0^{aA}	87.1 ± 9.3^{aA}	78.2 ± 9.7^{aA}	68.3 ± 7.6^{aA}	50.5 ± 11.6^{aB}	8.9 ± 2.0^{bB}	7.5 ± 0.5^{bB}	6.5 ± 0.3^{bB}	7.3 ± 0.6^{bB}
LIN (%)	39.9 ± 1.6^{bA}	47.4 ± 2.1^{abA}	49.8 ± 1.7^{aA}	47.7 ± 1.9^{abA}	49.5 ± 2.3^{abA}	46.7 ± 3.7^{abA}	42.3 ± 2.6^{aA}	31.5 ± 3.1^{aB}	16.5 ± 2.8^{bB}	15.4 ± 2^{bB}	10.2 ± 1.4^{cB}	13.8 ± 1.5^{bcB}
STR (%)	54.8 ± 1.7^{aA}	59.2 ± 2.5^{aA}	62.3 ± 1.7^{aA}	59.7 ± 1.9^{aA}	61.3 ± 2.1^{aA}	61.9 ± 3.4^{aA}	57.5 ± 2.6^{aA}	51.8 ± 1.9^{abB}	43 ± 4.6^{bcB}	39.1 ± 2.7^{bcB}	30.3 ± 3.6^{cB}	35.2 ± 2.7^{cB}
WOB (%)	70.8 ± 2.3^{aA}	77.9 ± 1.5^{aA}	76.7 ± 1.2^{aA}	77.5 ± 1.2^{aA}	77.2 ± 1.5^{aA}	72 ± 2.7^{aA}	69.5 ± 1.8^{aA}	59.6 ± 3.3^{aB}	35.4 ± 3.1^{bB}	36.3 ± 2.3^{bB}	32.4 ± 2^{bB}	37.6 ± 4.2^{bB}
ALH (µm)	0.61 ± 0.03^{aA}	0.65 ± 0.05^{aA}	0.55 ± 0.02^{abA}	0.60 ± 0.03^{aA}	0.53 ± 0.03^{bA}	0.55 ± 0.04^{bA}	0.52 ± 0.02^{aB}	0.56 ± 0.02^{aA}	0.40 ± 0.02^{bB}	0.40 ± 0.02^{bB}	0.40 ± 0.02^{bB}	0.41 ± 0.02^{bB}
BCF [Hz]	9.8 ± 0.5^{aA}	8.8 ± 0.6^{aA}	9.4 ± 0.4^{aA}	9.0 ± 0.4^{aA}	9.1 ± 0.6^{aA}	8.7 ± 0.6^{aA}	8.0 ± 0.4^{aB}	9.2 ± 0.7^{aA}	3.9 ± 0.6^{bB}	3.3 ± 0.5^{bB}	2.9 ± 0.5^{bB}	3.0 ± 0.4^{bB}

Data are displayed as mean ± standard error. Total motility (TM; VCL ≥ 15 µm/sec); Progressive motility (PM; 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; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency. Different lowercase letters indicate a significant difference between pH treatments within an incubation period, and different capital letters indicate a significant difference between different incubation periods at the same pH ($P < 0.05$).

Appendix E Motility parameters of barramundi, *Lates calcarifer*, (n = 7) testicular spermatozoa incubated at 4 °C for 1 h and 24 h undiluted (milt control) or diluted in HEPES buffered non-activating medium (NAM: 182.4 mM NaCl, 5.1 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 10 mM HEPES, 5.6 mM D^+ glucose) with different pH.

	1 h incubation							24 h incubation						
Parameter	**Control**	**pH 6.5B**	**pH 6.5H**	**pH 7.4**	**pH 7.8**	**pH 8.1**	**pH 8.5**	**Control**	**pH 6.5B**	**pH 6.5H**	**pH 7.4**	**pH 7.8**	**pH 8.1**	**pH 8.5**
TM (%)	49.6 ± 2.7^{aA}	50.7 ± 6.8^{aA}	48.7 ± 5.3^{aA}	51.8 ± 7.5^{aA}	55.8 ± 4.0^{aA}	45.9 ± 4.7^{aA}	49.2 ± 3.5^{aA}	28.1 ± 6.3^{aB}	7.1 ± 1.2^{bB}	9.3 ± 2.9^{bB}	35.1 ± 3.3^{aB}	29.7 ± 3.4^{aB}	8.1 ± 2.4^{bB}	8.4 ± 2.6^{bB}
PM (%)	22.6 ± 2.7^{aA}	28.8 ± 6.6^{aA}	25.9 ± 3.3^{aA}	31.1 ± 7.4^{aA}	29.8 ± 3.7^{aA}	24.9 ± 6.0^{aA}	24.9 ± 4.1^{aA}	10.4 ± 3.5^{aA}	1.7 ± 0.9^{bB}	3.3 ± 1.3^{bB}	18.8 ± 3.0^{aA}	13.0 ± 2.5^{aB}	1.2 ± 0.6^{bB}	2.1 ± 1.1^{bB}
Slow (%)	27.0 ± 1.4^{aA}	21.9 ± 1.0^{aA}	22.9 ± 2.7^{aA}	20.7 ± 1.7^{aA}	26.1 ± 3.1^{aA}	20.9 ± 2.6^{aA}	24.4 ± 2.6^{aA}	17.7 ± 2.9^{aB}	5.3 ± 0.7^{bB}	5.9 ± 1.7^{bB}	16.2 ± 2.1^{aA}	16.7 ± 2.3^{aA}	6.9 ± 1.8^{bB}	6.3 ± 1.5^{bB}
Medium (%)	3.5 ± 0.4^{aA}	1.9 ± 0.3^{aA}	2.4 ± 0.6^{aA}	2.3 ± 0.4^{aA}	2.7 ± 0.3^{aA}	1.7 ± 0.5^{aA}	1.9 ± 0.2^{aA}	1.8 ± 0.6^{abA}	0.5 ± 0.2^{bcB}	0.8 ± 0.2^{abcB}	2.1 ± 0.2^{aA}	1.1 ± 0.3^{abcA}	0.2 ± 0.1^{cA}	0.4 ± 0.2^{bcA}
Fast (%)	19.1 ± 2.7^{aA}	26.8 ± 6.3^{aA}	23.5 ± 2.9^{aA}	28.9 ± 7.3^{aA}	27.1 ± 3.8^{aA}	23.2 ± 6.2^{aA}	23.0 ± 4.1^{aA}	8.6 ± 3.0^{aA}	1.2 ± 0.9^{bB}	2.6 ± 1.1^{bB}	16.8 ± 2.9^{aA}	11.8 ± 2.5^{aA}	1.0 ± 0.5^{bB}	1.7 ± 1.1^{bB}
VCL (μm/s)	81.5 ± 7.8^{aA}	98.6 ± 8.7^{aA}	102.5 ± 3.7^{aA}	110.0 ± 10.5^{aA}	108.0 ± 7.9^{aA}	102.3 ± 15.5^{aA}	105.8 ± 8.6^{aA}	64.9 ± 6.6^{abA}	36.9 ± 9.1^{cB}	45.9 ± 8.2^{bcB}	86.0 ± 8.6^{aA}	83.2 ± 9.2^{aA}	34.6 ± 6.1^{cB}	35.1 ± 8.2^{cB}
VSL (μm/s)	49.4 ± 7.1^{aA}	46.8 ± 6.5^{aA}	50.2 ± 7.1^{aA}	65.1 ± 5.7^{aA}	61.0 ± 3.7^{aA}	49.2 ± 9.6^{aA}	64.3 ± 7.1^{aA}	33.5 ± 6.2^{abcA}	9.7 ± 2.6^{cB}	18.7 ± 7.0^{abcA}	38.1 ± 6.4^{abB}	43.1 ± 7.8^{aA}	12.1 ± 4.0^{bcB}	13.7 ± 6.7^{bcB}
VAP (μm/s)	71.5 ± 8.6^{aA}	87.5 ± 8.3^{aA}	92.4 ± 4.1^{aA}	100.5 ± 10.0^{aA}	97.9 ± 7.4^{aA}	88.8 ± 15.4^{aA}	93.9 ± 8.7^{aA}	53.9 ± 7.5^{abA}	23.8 ± 9.5^{cB}	33.4 ± 9.0^{bcB}	74.7 ± 9.1^{aA}	72.0 ± 9.5^{aA}	22.4 ± 6.3^{cB}	22.8 ± 9.0^{cB}
LIN (%)	44.1 ± 3.7^{aA}	39.1 ± 2.9^{aA}	40.5 ± 4.1^{aA}	47.2 ± 3.5^{aA}	45.2 ± 1.3^{aA}	37 ± 2.5^{aA}	43.2 ± 3.8^{aA}	38.3 ± 3.9^{aA}	21 ± 2.8^{bcB}	24.4 ± 2.4^{abcB}	34.9 ± 3.6^{abcB}	36.9 ± 3.4^{abB}	24.6 ± 4.9^{abcA}	20 ± 4.6^{cA}
STR (%)	60.3 ± 3^{aA}	51.5 ± 3^{aA}	53.1 ± 4.6^{aA}	59.2 ± 3.5^{aA}	58.4 ± 1.7^{aA}	51.3 ± 2^{aA}	56.6 ± 3^{aA}	56.6 ± 3.2^{aA}	43.8 ± 2.2^{aB}	43.5 ± 3.3^{aA}	48.2 ± 3^{aB}	52.6 ± 2.6^{aA}	48.5 ± 6.3^{aA}	39.5 ± 5.3^{aA}
WOB (%)	67.8 ± 2.8^{aA}	72 ± 2.4^{aA}	73 ± 2^{aA}	75.1 ± 2.3^{aA}	72.9 ± 1.9^{aA}	68.2 ± 3.6^{aA}	68.9 ± 3.4^{aA}	62.2 ± 3.1^{abcA}	45.4 ± 6.4^{bcA}	50.8 ± 4.6^{abcB}	69.4 ± 3.8^{aA}	64.6 ± 3.2^{abA}	45.1 ± 4.9^{bcB}	43.1 ± 5^{cA}
ALH (μm)	0.51 ± 0.01^{aA}	0.63 ± 0.04^{aA}	0.62 ± 0.04^{aA}	0.59 ± 0.04^{aA}	0.59 ± 0.03^{aA}	0.63 ± 0.04^{aA}	0.60 ± 0.04^{aA}	0.52 ± 0.02^{abA}	0.46 ± 0.04^{bcA}	0.45 ± 0.03^{bcA}	0.56 ± 0.03^{aA}	0.56 ± 0.02^{aB}	0.45 ± 0.03^{cB}	0.42 ± 0.02^{cB}
BCF [Hz]	8.5 ± 0.4^{aA}	6.7 ± 0.9^{aA}	6.7 ± 0.6^{aA}	8.5 ± 0.5^{aA}	8.0 ± 0.6^{aA}	8.5 ± 0.9^{aA}	8.1 ± 1.0^{aA}	6.6 ± 0.2^{abB}	4.2 ± 0.4^{bcB}	6.8 ± 0.9^{aA}	8.3 ± 0.7^{aA}	7.1 ± 0.6^{aA}	3.7 ± 0.7^{cB}	4.0 ± 0.4^{cA}

Data are displayed as mean ± standard error; Total motility (TM; VCL ≥ 15 μm/s); Progressive motility (PM; 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; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency. Different lowercase letters indicate a significant difference between pH treatments within an incubation period, and different capital letters indicate a significant difference between different incubation periods at the same pH (P < 0.05).

Appendix F Motility parameters of barramundi, *Lates calcarifer*, (n = 6) testicular spermatozoa incubated at 4 °C for 1 h and 24 h undiluted (milt control) or diluted in HEPES buffered non-activating medium (NAM: 0-190 mM NaCl, 0-190 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 10 mM HEPES, and 5.6 mM D^+ glucose at pH 7.4 and 400 mOsm/kg) containing different NaCl/KCl concentrations.

	1 h incubation						24 h incubation					
Parameter	**Control**	**0 mM NaCl/ 190 mM KCl**	**140 mM NaCl/ 50 mM KCl**	**160 mM NaCl/ 30 mM KCl**	**185 mM NaCl/ 5 mM KCl**	**190 mM NaCl/ 0 mM KCl**	**Control**	**0 mM NaCl/ 190 mM KCl**	**140 mM NaCl/ 50 mM KCl**	**160 mM NaCl/ 30 mM KCl**	**185 mM NaCl/ 5 mM KCl**	**190 mM NaCl/ 0 mM KCl**
TM (%)	43.5 ± 4.5^{aA}	4.6 ± 0.6^{bA}	47.1 ± 4.8^{aA}	44.5 ± 3.5^{aA}	56.8 ± 5.0^{aA}	56.5 ± 4.3^{aA}	24.8 ± 6.7^{abcB}	8.8 ± 5.1^{cA}	21.9 ± 5.3^{bcB}	18.4 ± 3.4^{bcB}	47.7 ± 7.2^{aA}	27.7 ± 1.5^{abB}
PM (%)	17.4 ± 2.3^{aA}	0.4 ± 0.2^{bA}	22.4 ± 3.1^{aA}	21.1 ± 2.5^{aA}	28.5 ± 2.4^{aA}	25.3 ± 3.1^{aA}	5.8 ± 1.9^{abB}	3.8 ± 3.8^{bA}	7.8 ± 3.1^{abB}	6.0 ± 1.8^{abB}	20.3 ± 3.6^{aB}	10.4 ± 1.8^{abB}
Slow (%)	26.1 ± 2.6^{aA}	4.3 ± 0.6^{bA}	24.8 ± 2.6^{aA}	23.4 ± 1.7^{aA}	28.3 ± 4.3^{aA}	31.2 ± 1.9^{aA}	19.0 ± 4.8^{abA}	4.9 ± 1.4^{cA}	14.0 ± 2.3^{bcA}	12.4 ± 1.8^{bcB}	27.3 ± 3.9^{aA}	17.3 ± 0.6^{abB}
Medium (%)	2.3 ± 0.4^{aA}	0.2 ± 0.1^{bA}	2.0 ± 0.6^{aA}	2.3 ± 0.3^{aA}	2.1 ± 0.3^{aA}	2.8 ± 0.5^{aA}	0.9 ± 0.4^{abA}	0.1 ± 0.1^{bA}	1.0 ± 0.5^{abA}	1.6 ± 0.5^{abA}	2.7 ± 0.5^{aA}	1.7 ± 0.3^{aA}
Fast (%)	15.1 ± 2.2^{bA}	0.2 ± 0.2^{cA}	20.4 ± 3.1^{abcA}	18.8 ± 2.6^{abA}	26.4 ± 2.3^{aA}	22.5 ± 3.0^{abA}	4.9 ± 1.6^{abB}	3.7 ± 3.7^{bA}	6.9 ± 2.6^{abB}	4.4 ± 1.3^{abB}	17.6 ± 3.3^{aB}	8.7 ± 1.7^{abB}
VCL (µm/s)	77.9 ± 6.8^{bA}	25.4 ± 5.3^{cA}	90.9 ± 8.1^{abA}	95.6 ± 7.0^{abA}	112.1 ± 7.4^{aA}	99.3 ± 6.9^{abA}	47.5 ± 6.1^{bcB}	42.6 ± 20.8^{cA}	54.8 ± 11.0^{abcB}	52.1 ± 6.3^{bcB}	78.8 ± 5.6^{aB}	64.3 ± 4.8^{abB}
VSL (µm/s)	48.2 ± 7.3^{bA}	6.9 ± 4.4^{cA}	61.4 ± 7.6^{abA}	65.8 ± 6.6^{abA}	79.1 ± 6.8^{aA}	62.5 ± 6.8^{abA}	26.4 ± 5.5^{abcB}	20.8 ± 18.1^{cA}	31.0 ± 8.0^{abB}	26.8 ± 5.2^{abcB}	42.7 ± 4.3^{aB}	25.7 ± 2.5^{bcB}
VAP (µm/s)	67.1 ± 7.0^{bA}	12.1 ± 4.8^{cA}	81.8 ± 8.4^{abA}	86.5 ± 7.2^{abA}	102.6 ± 7.9^{aA}	87.9 ± 7.2^{abA}	35.9 ± 6.5^{bcB}	29.3 ± 21.8^{cA}	45.5 ± 11.3^{abB}	41.0 ± 6.8^{bcB}	67.1 ± 5.8^{aB}	50.5 ± 4.2^{abB}
LIN (%)	41.5 ± 2.9^{aA}	15.6 ± 3.3^{bA}	48.2 ± 2.4^{aA}	48 ± 2.6^{aA}	50.1 ± 3.8^{aA}	44.6 ± 3.7^{aA}	31.6 ± 4.1^{abA}	19.4 ± 8^{bA}	37.1 ± 4.4^{aB}	33.6 ± 3.5^{aA}	38.5 ± 2.9^{aB}	29 ± 2.3^{abB}
STR (%)	59.2 ± 3^{aA}	35.4 ± 5^{bA}	63.1 ± 1.3^{aA}	62.2 ± 2.5^{aA}	63.6 ± 3.1^{aA}	60.6 ± 3.7^{aA}	51.9 ± 3.9^{abA}	38.8 ± 6.3^{bA}	55.8 ± 3.1^{aB}	50.9 ± 2.9^{abA}	55.1 ± 3.5^{aB}	47.6 ± 2.9^{abA}
WOB (%)	63.5 ± 1.2^{aA}	38.7 ± 2.3^{bA}	69.1 ± 2.2^{aA}	69.8 ± 1.9^{aA}	71.1 ± 3.1^{aA}	67.1 ± 1.8^{aA}	52.1 ± 3.3^{bcB}	42.1 ± 7.8^{cA}	59.5 ± 5^{abB}	57.8 ± 3.4^{abcA}	63.8 ± 2^{aA}	57.3 ± 2.2^{abcA}
ALH (µm)	0.51 ± 0.02^{aA}	0.40 ± 0.01^{bA}	0.50 ± 0.02^{aA}	0.50 ± 0.01^{aA}	0.55 ± 0.02^{aA}	0.59 ± 0.04^{aA}	0.45 ± 0.02^{abB}	0.45 ± 0.03^{abA}	0.43 ± 0.02^{bB}	0.46 ± 0.02^{abB}	0.56 ± 0.04^{aA}	0.55 ± 0.03^{abA}
BCF [Hz]	7.9 ± 0.5^{abA}	3.3 ± 1.2^{cA}	8.2 ± 0.6^{abA}	8.8 ± 0.4^{aA}	8.8 ± 0.8^{abA}	7.4 ± 0.3^{bcA}	5.4 ± 0.4^{abB}	3.5 ± 1.2^{bA}	6.7 ± 1.0^{aA}	6.7 ± 0.7^{aA}	6.8 ± 0.2^{aA}	6.8 ± 0.5^{aA}

Data are displayed as mean ± standard error. Total motility (TM; VCL ≥ 15 µm/s); Progressive motility (PM; 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; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency. Different lowercase letters indicate a significant difference between concentration treatments within an incubation period, and different capital letters indicate a significant difference between incubation periods at the same concentration treatment ($P < 0.05$).

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Appendix G Motility parameters of barramundi, *Lates calcarifer*, (n = 6) testicular spermatozoa incubated at 4 °C for up to 96 h undiluted (milt control) or diluted in HEPES buffered non-activating medium (NAM: 185 mM NaCl, 5.0 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 10 mM HEPES and 5.6 mM D^+ glucose at pH 7.4 and 400 mOsm/kg).

	1 h incubation		24 h incubation		48 h incubation		72 h incubation		96 h incubation	
Parameter	**Control**	**185 mM NaCl/ 5 mM KCl**	**Control**	**185 mM NaCl/ 5 mM KCl**	**Control**	**185 mM NaCl/ 5 mM KCl**	**Control**	**185 mM NaCl/ 5 mM KCl**	**Control**	**185 mM NaCl/ 5 mM KCl**
TM (%)	43.5 ± 4.5^{bA}	56.8 ± 5.0^{aA}	24.8 ± 6.7^{aAB}	47.7 ± 7.2^{aA}	12.6 ± 4.8^{aBC}	29.3 ± 9.3^{aAB}	7.4 ± 2.8^{aBC}	16.9 ± 6.7^{aB}	3.1 ± 0.7^{aC}	3.1 ± 0.6^{aB}
PM (%)	17.4 ± 2.3^{bA}	28.5 ± 2.4^{aA}	5.8 ± 1.9^{bB}	20.3 ± 3.6^{aAB}	4.4 ± 2.6^{aB}	9.8 ± 3.4^{aBC}	2.2 ± 1.5^{aB}	5.7 ± 2.6^{aC}	0.0 ± 0.0^{aC}	0.2 ± 0.1^{aC}
Slow (%)	26.1 ± 2.6^{aA}	28.3 ± 4.3^{aA}	19.0 ± 4.8^{aAB}	27.3 ± 3.9^{aA}	8.2 ± 2.5^{aBC}	19.5 ± 6.0^{aAB}	5.2 ± 1.5^{aBC}	11.1 ± 4.3^{aAB}	3.1 ± 0.7^{aC}	2.9 ± 0.5^{aB}
Medium (%)	2.3 ± 0.4^{aA}	2.1 ± 0.3^{aA}	0.9 ± 0.4^{bAB}	2.7 ± 0.5^{aA}	0.4 ± 0.2^{aB}	1.8 ± 0.6^{aAB}	0.2 ± 0.1^{aB}	0.5 ± 0.3^{aAB}	0.0 ± 0.0^{aB}	0.2 ± 0.1^{aB}
Fast (%)	15.1 ± 2.2^{bA}	26.4 ± 2.3^{aA}	4.9 ± 1.6^{bB}	17.6 ± 3.3^{aB}	4.0 ± 2.6^{aAB}	8.0 ± 3.0^{aBC}	1.9 ± 1.6^{aBC}	5.2 ± 2.4^{aC}	0.0 ± 0.0^{aC}	0.0 ± 0.0^{aC}
VCL (µm/s)	77.9 ± 6.8^{bA}	112.1 ± 7.4^{aA}	47.5 ± 6.1^{bB}	78.8 ± 5.6^{aB}	60.8 ± 17.0^{aAB}	56.3 ± 15.3^{aBC}	38.5 ± 12.0^{aAB}	55.2 ± 12.9^{aBC}	19.6 ± 0.9^{aC}	20.1 ± 1.4^{aC}
VSL (µm/s)	48.2 ± 7.3^{bA}	79.1 ± 6.8^{aA}	26.4 ± 5.5^{aA}	42.7 ± 4.3^{aB}	32.7 ± 13.1^{aAB}	35.1 ± 15.6^{aABC}	14.8 ± 10.5^{aAB}	25.1 ± 10.0^{aBC}	2.2 ± 0.9^{aB}	1.9 ± 0.7^{aC}
VAP (µm/s)	67.1 ± 7.0^{bA}	102.6 ± 7.9^{aA}	35.9 ± 6.5^{bB}	67.1 ± 5.8^{aB}	49.1 ± 18.4^{aABC}	44.4 ± 16.1^{aBC}	24.4 ± 13.2^{aABC}	44.7 ± 13.4^{aBC}	6.5 ± 1.1^{aC}	6.1 ± 1.2^{aC}
LIN (%)	41.5 ± 2.9^{bA}	50.1 ± 3.8^{aA}	31.6 ± 4.1^{aA}	38.5 ± 2.9^{aA}	28.7 ± 7.2^{aAB}	32.5 ± 9.1^{aAB}	19.2 ± 6.2^{aAB}	27.4 ± 7.4^{aAB}	10.1 ± 3.1^{aB}	7.3 ± 1.5^{aB}
STR (%)	59.2 ± 3^{aA}	63.6 ± 3.1^{aA}	51.9 ± 3.9^{aA}	55.1 ± 3.5^{aA}	44.4 ± 7.5^{aAB}	49.5 ± 10.1^{aAB}	39.7 ± 4.4^{aAB}	44.5 ± 7.3^{aAB}	26.8 ± 2.8^{aB}	24.6 ± 2.8^{aB}
WOB (%)	63.5 ± 1.2^{bA}	71.1 ± 3.1^{aA}	52.1 ± 3.3^{aB}	63.8 ± 2^{aA}	51.1 ± 8^{aABC}	52.3 ± 7.9^{aAB}	41.4 ± 6.6^{aABC}	52.5 ± 8.4^{aAB}	31.7 ± 4.1^{aC}	27.6 ± 2.7^{aB}
ALH (µm)	0.51 ± 0.02^{aA}	0.55 ± 0.02^{aA}	0.45 ± 0.02^{bA}	0.56 ± 0.04^{aA}	0.46 ± 0.04^{aAB}	0.51 ± 0.07^{aAB}	0.44 ± 0.02^{aAB}	0.45 ± 0.04^{aAB}	0.34 ± 0.03^{aB}	0.36 ± 0.03^{aB}
BCF [Hz]	7.9 ± 0.5^{aA}	8.8 ± 0.8^{aA}	5.4 ± 0.4^{aB}	6.8 ± 0.2^{aA}	5.3 ± 1.2^{aABC}	5.5 ± 1.5^{aAB}	4.5 ± 0.8^{aBC}	4.6 ± 1.0^{aB}	1.8 ± 0.4^{aC}	1.9 ± 0.3^{aB}

Data are displayed as mean ± standard error. Total motility (TM; VCL ≥ 15 µm/s); Progressive motility (PM; 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; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency. Different lowercase letters indicate a significant difference between extender treatments within an incubation period, and different capital letters indicate a significant difference between incubation periods for the same medium($P < 0.05$).

Appendix H Total motility of barramundi, *Lates calcarifer*, (n = 6) testicular spermatozoa incubated at 4 ℃ for up to 96 h undiluted (milt control) or diluted in HEPES buffered non-activating medium (NAM: 185 mM NaCl, 5.0 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 10 mM HEPES, and 5.6 mM D^+ glucose at pH 7.4 and 400 mOsm/kg).

	1 h incubation		24 h incubation		48 h incubation		72 h incubation		96 h incubation	
Male	**Control**	**185 mM NaCl/ 5 mM KCl**	**Control**	**185 mM NaCl/ 5 mM KCl**	**Control**	**185 mM NaCl/ 5 mM KCl**	**Control**	**185 mM NaCl/ 5 mM KCl**	**Control**	**185 mM NaCl/ 5 mM KCl**
1	40.1 ± 7.5	54.4 ± 2.4	10.3 ± 0.7	16.3 ± 1.6	3.2 ± 1.1	2.0 ± 0.8	2.1 ± 1.0	0.0 ± 0.0	1.9 ± 0.4	2.1 ± 0.7
2	41.9 ± 3.5	51.2 ± 4.7	18.6 ± 2.0	49.4 ± 5.4	3.5 ± 1.4	2.9 ± 1.4	3.7 ± 1.1	3.2 ± 1.2	6.2 ± 0.6	3.2 ± 1.1
3	46.6 ± 2.1	62.5 ± 2.1	34.4 ± 6.0	59.1 ± 2.2	9.7 ± 2.1	25.3 ± 3.2	1.7 ± 0.6	3.0 ± 2.2	1.0 ± 0.5	4.1 ± 0.8
4	61.7 ± 2.9	65.8 ± 3.2	48.1 ± 3.1	66.8 ± 0.3	19.1 ± 3.5	49.4 ± 5.9	11.5 ± 2.8	35.4 ± 3.5	3.8 ± 1.2	5.3 ± 0.8
5	43.2 ± 3.8	70.4 ± 2.4	32.7 ± 1.1	53.2 ± 2.5	6.4 ± 1.9	51.8 ± 4.1	6.4 ± 2.1	31.8 ± 2.5	2.7 ± 0.4	2.8 ± 0.3
6	27.9 ± 3.8	36.8 ± 6.0	4.6 ± 0.9	41.2 ± 1.2	33.5 ± 3.3	44.1 ± 1.1	19.1 ± 1.9	27.7 ± 5.2	2.8 ± 1.0	1.3 ± 1.3
Mean	43.5 ± 4.5	56.8 ± 5.0	24.8 ± 6.7	47.7 ± 7.2	12.6 ± 4.8	29.3 ± 9.3	7.4 ± 2.8	16.9 ± 6.7	3.1 ± 0.7	3.1 ± 0.6

Data are displayed as mean ± standard error. Total motility (VCL ≥ 15 μm/s).

Appendix I Physical, milt and sperm quality characteristics for each male barramundi (*Lates calcarifer*) broodstock (*n* = 22).

ID	BW	TL	K	Vol.	Conc.	TC	TM	PM	Slow	Med.	Fast	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	Live	DNA
M1	3.9	73	1.00	63.9	31.7 ± 0.54	2023.3	40.2 ± 5.4	25.5 ± 3.7	14.8 ± 2.1	11.5 ± 2.3	13.8 ± 2.5	84.9 ± 5.4	36.2 ± 7.1	75.4 ± 5.3	43.0 ± 6.0	55.0 ± 6.0	76.0 ± 0.0	0.57 ± 0.04	8.7 ± 0.6	90.8 ± 0.3	0.2 ± 0.0
M2	3.6	61	1.59	5.8	6.0 ± 0.40	35.0	70.1 ± 2.3	60 ± 7.8	10.1 ± 5.6	13.9 ± 4	46.1 ± 10.6	152.9 ± 27.2	128.3 ± 24.3	147.3 ± 29.2	74.0 ± 6.0	82.0 ± 3.0	87.0 ± 6.0	0.48 ± 0.01	13.8 ± 0.7	89.9 ± 1.8	1.8 ± 0.3
M3	3.9	71	1.09	23.2	15.2 ± 0.82	354.1	53.6 ± 4.3	38.5 ± 4.1	15.2 ± 2	11.7 ± 1.1	26.3 ± 5.3	117.1 ± 6.0	79.1 ± 1.7	108.1 ± 5.4	57.0 ± 2.0	68.0 ± 2.0	80.0 ± 1.0	0.54 ± 0.03	11.9 ± 0.8	92.8 ± 0.3	5.6 ± 0.2
M4	3.4	70	0.99	17.4	5.5 ± 0.80	96.1	13.4 ± 1.3	12.4 ± 0.3	1.1 ± 1.1	8.1 ± 1	4.3 ± 1.2	98.1 ± 8.1	50.7 ± 14.7	86.7 ± 13.4	57.0 ± 12.0	68.0 ± 8.0	84.0 ± 7.0	0.51 ± 0.06	15.3 ± 2.8	75.8 ± 0.3	6.7 ± 0.1
M5	4.8	76	1.09	5.8	9.4 ± 1.09	54.4	20.6 ± 3.9	13.2 ± 2.3	7.4 ± 2.7	8.1 ± 3.1	5.1 ± 1.9	76.4 ± 5.6	48.8 ± 5.7	68.2 ± 6.6	59.0 ± 6.0	70.0 ± 5.0	80.0 ± 5.0	0.51 ± 0.03	12.0 ± 0.6	81.8 ± 0.2	19.3 ± 0.3
M6	4.2	75	1.00	3.5	11.9 ± 0.59	41.3	28.9 ± 3.5	21.3 ± 2.2	7.6 ± 1.4	11.4 ± 2.4	9.9 ± 1.1	98.3 ± 8.5	65.8 ± 5.8	90.9 ± 9.8	58.0 ± 4.0	70.0 ± 2.0	81.0 ± 4.0	0.50 ± 0.01	13.1 ± 1.1	83.3 ± 0.5	24.7 ± 0.1
M7	4.9	75	1.16	9.9	22.3 ± 1.93	220.5	16.7 ± 1.9	9.2 ± 1.1	7.5 ± 1.5	3.3 ± 0.6	5.9 ± 0.7	84.8 ± 4.9	45.7 ± 6.1	77.0 ± 4.9	50.0 ± 7.0	61.0 ± 7.0	77.0 ± 1.0	0.51 ± 0.01	9.1 ± 1.4	77.7 ± 0.1	58.9 ± 0.9
M8	3.2	64.5	1.19	14.5	12.4 ± 1.81	179.8	34.1 ± 3.3	26.1 ± 1.1	8 ± 3.3	14.7 ± 1.5	11.5 ± 1.2	91.1 ± 11.6	66.0 ± 12.0	86.7 ± 12.4	70.0 ± 3.0	79.0 ± 1.0	88.0 ± 3.0	0.44 ± 0.01	11.2 ± 0.6	91.0 ± 0.2	8.3 ± 0.6
M9	2.7	66	0.94	49.4	25.9 ± 1.05	1279.0	49.4 ± 2.7	36.4 ± 3.6	13 ± 3.4	16.9 ± 1.4	19.3 ± 3.1	76.7 ± 5.5	26.6 ± 3.3	60.4 ± 5.6	36.0 ± 3.0	53.0 ± 5.0	68.0 ± 2.0	0.62 ± 0.03	12.2 ± 0.3	87.8 ± 0.4	0.0 ± 0.0
M10	3.3	69	1.00	41.3	18.6 ± 0.83	765.3	32.2 ± 3	22.8 ± 2.9	9.4 ± 1.1	13.8 ± 2	8.6 ± 1.8	87.9 ± 8.3	25.4 ± 4.1	72.4 ± 7.4	34.0 ± 4.0	48.0 ± 4.0	73.0 ± 2.0	0.67 ± 0.06	11.6 ± 1.0	85.9 ± 0.2	0.1 ± 0.0
M11	3.6	70	1.05	15.7	23.5 ± 2.12	368.3	28.7 ± 1.5	18.7 ± 1.8	10 ± 1.4	13 ± 1.2	5.5 ± 1	75.3 ± 7.4	33.3 ± 4.2	65.2 ± 7.7	48.0 ± 1.0	59.0 ± 1.0	79.0 ± 2.0	0.56 ± 0.02	11.0 ± 0.7	73.6 ± 0.5	0.0 ± 0.0
M12	3.3	70	0.96	49.4	21.5 ± 2.04	1060.9	47.4 ± 2	35.8 ± 2.1	11.6 ± 1.1	21.2 ± 2.5	13.8 ± 1.9	69.4 ± 6.0	29.8 ± 1.8	56.1 ± 5.9	42.0 ± 2.0	59.0 ± 3.0	69.0 ± 2.0	0.56 ± 0.02	10.4 ± 0.5	78.4 ± 0.1	0.3 ± 0.0
M13	1.9	59	0.93	61	27.0 ± 1.82	1645.2	35.7 ± 2.4	25 ± 2	10.7 ± 0.9	15.4 ± 1.8	9.4 ± 1.2	83.9 ± 6.4	28.7 ± 6.6	68.3 ± 7.5	35.0 ± 3.0	50.0 ± 3.0	70.0 ± 2.0	0.64 ± 0.03	11.3 ± 0.8	84.6 ± 0.2	0.1 ± 0.0
M14	2.3	67	0.76	32	20.4 ± 0.72	652.7	36.6 ± 4.9	26.1 ± 4.7	10.5 ± 1.3	12.5 ± 2.4	13.5 ± 3	74.4 ± 4.0	22.4 ± 2.4	60.0 ± 4.1	33.0 ± 2.0	48.0 ± 3.0	70.0 ± 1.0	0.59 ± 0.03	12.2 ± 0.8	71.9 ± 0.3	0.1 ± 0.0
M15	2.7	70	0.79	22.7	21.8 ± 1.81	493.4	18.4 ± 2.4	9.5 ± 1.8	8.9 ± 0.7	7.1 ± 1	2.4 ± 1	89.7 ± 10.0	42.5 ± 5.9	76.7 ± 10.5	39.0 ± 2.0	54.0 ± 3.0	71.0 ± 3.0	0.58 ± 0.03	11.2 ± 0.3	80.7 ± 0.2	0.0 ± 0.0
M16	3.2	66.5	1.09	23.8	23.2 ± 1.72	551.5	48.3 ± 3.1	32.9 ± 1.2	15.4 ± 2.7	19.6 ± 1.5	13.3 ± 1.7	52.2 ± 5.9	19.6 ± 3.6	36.2 ± 6.3	35.0 ± 3.0	55.0 ± 3.0	60.0 ± 4.0	0.54 ± 0.03	10.8 ± 0.4	60.7 ± 0.1	0.0 ± 0.0
M17	3.8	73	0.98	37.8	12.9 ± 0.36	489.0	22.7 ± 2.1	12.7 ± 0.5	10 ± 1.8	7.6 ± 1.5	5.1 ± 2	60.6 ± 4.5	38.9 ± 5.0	49.2 ± 5.6	49.0 ± 2.0	70.0 ± 5.0	66.0 ± 1.0	0.45 ± 0.03	11.6 ± 0.9	85.9 ± 0.1	7.2 ± 0.1
M18	4.3	76.5	0.96	29.1	17.1 ± 1.50	497.5	44.6 ± 3.3	36.2 ± 3.2	8.4 ± 0.4	25 ± 2	11.2 ± 1.2	77.6 ± 0.9	52.7 ± 2.8	65.1 ± 1.0	58.0 ± 3.0	76.0 ± 3.0	73.0 ± 1.0	0.50 ± 0.00	16.1 ± 0.9	93.0 ± 0.5	1.1 ± 0.3
M19	3.5	74.5	0.85	61	26.3 ± 1.56	1607.4	31.9 ± 3.1	20 ± 1.7	11.9 ± 1.4	15.6 ± 1.9	4.4 ± 0.6	57.0 ± 3.1	41.1 ± 3.9	49.6 ± 3.7	63.0 ± 2.0	76.0 ± 2.0	78.0 ± 2.0	0.42 ± 0.01	11.9 ± 0.4	91.9 ± 0.2	0.1 ± 0.0
M20	3.2	76.5	0.71	37.8	25.7 ± 1.93	971.4	38.2 ± 2.1	26.8 ± 2.2	11.4 ± 1.6	18.4 ± 1.3	8.2 ± 1.3	70.8 ± 6.7	50.9 ± 5.9	62.0 ± 6.8	63.0 ± 1.0	77.0 ± 0.0	78.0 ± 1.0	0.46 ± 0.02	13.7 ± 0.6	93.8 ± 0.3	0.1 ± 0.0
M21	3.1	69	0.94	52.3	19.8 ± 1.60	1034.2	42 ± 2.6	32.5 ± 1.5	9.5 ± 1.3	20.9 ± 2.4	11.2 ± 1.7	81.1 ± 5.4	57.6 ± 5.6	72.5 ± 6.4	63.0 ± 3.0	76.0 ± 2.0	80.0 ± 2.0	0.47 ± 0.01	13.5 ± 0.6	93.0 ± 0.3	2.4 ± 0.0
M22	3.3	69	1.00	69.7	24.0 ± 2.04	1672.0	37.5 ± 1.2	29.6 ± 1.8	7.9 ± 1.2	20.3 ± 1.7	9.1 ± 2	72.7 ± 5.6	43.0 ± 5.6	59.9 ± 6.9	54.0 ± 4.0	71.0 ± 2.0	74.0 ± 3.0	0.49 ± 0.01	15.7 ± 0.9	91.6 ± 0.3	5.0 ± 0.8

Data are displayed as mean ± standard error; ID, fish identification number; BW, body weight (kg); TL, total length (cm); K, relation condition factor K; Vol., milt volume (µL); Conc., sperm concentration (x 10^9 sp/mL); TC, sperm total count (x 10^6); TM, total motility (VCL ≥ 15 µm/s); PM, 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, amplitude of lateral head displacement (µm); BCF, beat cross frequency (Hz); Live, proportion of viable sperm (%); DNA, proportion of DNA damage sperm (%).

Appendix J Summary of genetic diversity metrics of offspring generated from barramundi (*Lates calcarifer*) broodstock cohorts held in Tanks A, B, and C across two consecutive spawning nights, including the number (*n*) of families produced, the number of offspring genotyped, tested, and assigned, and measures of genetic diversity; number of sires which parented offspring (N_s), number of dams which parented offspring (N_d), mean numbers of offspring per sire (K_s) and dam (K_d), the variance in the contribution for sires (V_s) and dams (V_d), effective number of sires (N_{es}) and dams (N_{ed}).

	Tank A				**Tank B**				**Tank C**			
	Night 1		**Night 2**		**Night 1**		**Night 2**		**Night 1**		**Night 2**	
Parameter	**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**
Families (*n*)	9	-	15	11	11	19	22	23	13	9	9	9
Genotyped	94	-	94	94	94	94	94	94	94	94	94	94
Tested	91	-	93	93	92	92	93	90	92	94	88	89
Assigned	91	-	93	93	92	92	93	88	92	94	88	89
N_s	7	-	8	7	8	8	8	8	6	5	6	6
N_d	3	-	3	3	2	4	4	4	3	3	2	3
K_s	11.38	-	11.63	13.29	11.50	11.50	11.63	11.00	15.33	15.67	14.67	14.83
K_d	22.75	-	23.25	23.25	23.00	23.00	23.25	22.00	30.67	31.33	29.33	29.67
V_s	262.8	-	126.8	85.4	89.1	44.6	42.8	22.9	169.9	383.7	131.9	23.8
V_d	1835.6	-	984.9	1164.9	1826.0	987.3	454.3	268.0	17.3	1108.3	18001.3	2296.3
N_{es}	2.35	-	4.27	4.92	4.99	6.33	6.43	10.85	3.58	1.98	3.84	5.70
N_{ed}	0.66	-	1.06	0.95	0.44	1.40	2.20	2.62	3.01	1.42	0.64	0.83

Appendix K Contribution of barramundi (*Lates calcarifer*) broodstock to offspring collected at 2.5 h post-fertilization (hpf) and 24 h post-hatch (hph) from two consecutive spawning nights. Data are displayed in percentage. ID, fish identification number; F, female; M, male.

	Tank A					**Tank B**					**Tank C**			
	Night 1		**Night 2**			**Night 1**		**Night 2**			**Night 1**		**Night 2**	
ID	**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**	**ID**	**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**	**ID**	**at 2.5 hpf**	**at 24 hph**	**at 2.5 hpf**	**at 24 hph**
F1	0	-	0	0	F5	5.4	9.8	2.2	10.2	F9	34.8	72.3	88.6	95.5
F2	95.6	-	73.1	78.5	F6	0	4.3	10.2	8	F10	37.0	3.2	0	2.2
F3	2.2	-	23.7	19.4	F7	0	9.8	32.3	37.5	F11	28.3	24.5	11.4	2.2
F4	2.2	-	3.2	2.2	F8	94.6	76.2	53.8	44.3	-	-	-	-	-
Min	0	-	0	0	Min	0	4.3	2.2	8	Min	28.3	3.2	0	2.2
Max	95.6	-	73.1	78.5	Max	94.6	76.2	53.8	44.3	Max	37.0	72.3	88.6	95.5
CV	188.3	-	135.0	146.8	CV	185.9	136.7	94.4	74.4	CV	13.6	106.2	144.6	161.5
M1	52.7	-	4.3	4.3	M9	6.5	15.2	15.1	13.6	M17	6.5	4.3	10.2	18
M2	0	-	1.1	0	M10	17.4	15.2	3.2	2.3	M18	37	26.6	10.2	12.4
M3	8.8	-	26.9	25.8	M11	31.5	21.7	4.3	11.4	M19	7.6	2.1	42	27
M4	2.2	-	32.3	26.9	M12	10.9	10.9	22.6	21.6	M20	31.5	54.3	8	14.6
M5	1.1	-	4.3	5.4	M13	2.2	5.4	14	14.8	M21	14.1	12.3	19.3	12.4
M6	5.5	-	5.4	9.7	M14	21.7	21.7	18.3	11.4	M22	3.3	0	10.2	15.7
M7	6.6	-	5.4	10.8	M15	3.3	10.9	6.5	10.2	-	-	-	-	-
M8	23.1	-	20.4	17.2	M16	6.5	7.6	16.1	14.8	-	-	-	-	-
Min	0	-	1.1	0	Min	2.2	5.4	3.2	2.3	Min	3.3	0	8	12.4
Max	52.7	-	32.3	26.9	Max	31.5	21.7	22.6	21.6	Max	37	54.3	42	27
CV	142.4	-	135.0	146.8	CV	82.0	136.7	94.4	74.4	CV	13.6	106.2	144.6	161.5

Appendix L Genetic diversity estimates for each locus of barramundi (*Lates calcarifer*) broodstock; sample size (N_c), number of alleles (k), allelic richness (A_r), observed (H_o) and expected (H_e) heterozygosity, and the inbreeding coefficient (F_{is}).

	Tank A						**Tank B**						**Tank C**					
Locus	**N_c**	**k**	**A_r**	**H_o**	**H_e**	**F_{is}**	**N_c**	**k**	**A_r**	**H_o**	**H_e**	**F_{is}**	**N_c**	**k**	**A_r**	**H_o**	**H_e**	**F_{is}**
Lca003	12	3	2.84	0.833	0.565	-0.507*	12	2	2.00	0.750	0.477	-0.571	9	3	2.78	0.889	0.562	-0.580
Lca08	12	1	1.00	0	0	---	12	1	1.00	0.000	0.000	---	9	1	1.00	0.000	0.000	---
Lca016	12	3	2.17	0.167	0.163	-0.023	12	2	1.58	0.083	0.083	-0.000	8	3	2.75	0.250	0.241	-0.037
Lca20	12	3	2.94	0.75	0.583	-0.303	12	3	2.84	0.500	0.598	0.165	9	4	3.56	0.444	0.618	0.281
Lca21	12	5	4.70	0.583	0.714	0.189	12	5	4.75	0.583	0.773	0.245	9	5	4.92	0.889	0.806	-0.103
Lca040	12	3	2.97	0.583	0.562	-0.041	12	3	2.82	0.500	0.417	-0.200	9	3	2.78	0.556	0.444	-0.250
Lca057	12	4	3.97	0.917	0.757	-0.222	12	5	4.77	0.750	0.795	0.057	9	4	4.00	0.889	0.771	-0.153
Lca58	11	5	3.55	0.273	0.338	0.2	12	5	4.30	0.500	0.598	0.165	8	3	2.75	0.250	0.241	-0.037
Lca64	11	8	6.11	0.636	0.736	0.141	12	7	5.15	0.750	0.640	-0.172	7	4	4.00	0.857	0.738	-0.161
Lca69	12	2	2.00	0.5	0.391	-0.294	12	2	2.00	0.583	0.424	-0.375	8	2	1.99	0.250	0.232	-0.077
Lca70	12	4	3.17	0.75	0.598	-0.269	12	2	2.00	0.333	0.515	0.353	9	3	2.78	0.667	0.493	-0.352
Lca74	12	3	2.84	0.5	0.507	0.015	12	2	2.00	0.333	0.470	0.290	9	2	2.00	0.333	0.514	0.351
Lca98	12	4	3.56	0.667	0.612	-0.093	12	3	2.99	0.583	0.629	0.072	9	3	2.99	0.556	0.528	-0.053
Lca154	12	2	2.00	0.333	0.464	0.290	12	3	2.58	0.583	0.447	-0.305	9	3	2.78	0.556	0.444	-0.250
Lca178	12	3	2.98	0.667	0.594	-0.128	11	4	3.27	0.818	0.595	-0.374	8	4	3.75	0.750	0.643	-0.167
Lca371	12	3	2.83	0.417	0.467	0.113	12	2	1.99	0.417	0.341	-0.222	9	2	2.00	0.556	0.417	-0.333
Total		3.5	3.10	0.536	0.502	-0.068		3.2	2.88	0.504	0.488	-0.034		3.1	2.93	0.543	0.481	-0.130*

* indicate significant deviation from expected Hardy-Weinberg proportions ($P < 0.05$)