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REVIEWS IN Aquaculture

REVIEW

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

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Abstract

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

KEYWORDS
artificial fertilisation, decapod crustaceans, male fertility tools, sperm cryopreservation, sperm quality, subfertility

1 | INTRODUCTION

More than 50% of the world's seafood products presently come from aquaculture[1]; with crustaceans making up the bulk of seafood produced that typically command high values in the global market.[1,2] Production of crustaceans through aquaculture has been increasing over the past decades[1] and by 2018, total global aquaculture production reached 9.4 million tonnes and was valued at US$ 69.3 billion.

Reproductive efficiency in crustacean aquaculture is generally focused on the performance of female broodstock, with little attention given to male fertility.[3] However, several reports have shown that sperm quality, as well as spermatophore formation, could affect reproductive efficiency in decapod crustaceans.[4–10] Hence, screening male broodstock for fertility is critical to achieving high fertilisation rates and yielding good quality offspring. Such screening can detect the presence of sub- or infertile male broodstock in the hatchery, which may reduce production efficiency and raise operational costs.[11]

A direct way to determine male fertility is to evaluate sperm quality.[11,12] Sperm quality refers to the capacity of spermatozoa to fertilise eggs successfully and yield normal embryos.[13,14] Evaluation of sperm quality in crustaceans to date is limited to conventional methods that include light microscopy for sperm concentration and morphometry, as well as measurement of gonad and spermatophore weights.[15–21] Predictive biomarkers developed for assessing sperm quality in vertebrates such as plasma membrane integrity, acrosome reaction, mitochondrial function and DNA fragmentation, can be modified and applied to invertebrates.[22] Meanwhile, traditional parameters, such as sperm concentration and morphometry, can also be analysed using advanced diagnostic tools including Computer-Assisted Sperm Analysis (CASA) and/or flow cytometry, which can produce highly accurate and fast results.[13,14,22–24]

In parallel to the development of advance assessment tools for sperm quality, freezing of gametes, particularly spermatozoa, is a significant step to advance the aquaculture industry.[25,26] Sperm cryopreservation technology paves the way for controlling the timing of reproduction and enables the genetic diversity of broodstock to be maintained in frozen banks. Conservation of genetics from wild stocks is a critical component of breeding programmes in aquaculture, preventing potential loss of genetic diversity (and hence fitness) through under representation of founders over multiple generations of captive breeding.[26] Moreover, cryopreservation of spermatozoa in liquid nitrogen (LN_2) for extended periods of time guarantees year-round supply of high-quality spermatozoa with minimal effort and space, thereby reducing hatchery costs required for male broodstock maintenance, as well as facilitating selective breeding.[25,27]

In crustacean aquaculture, animals often only reach sexual maturity and reproduce in captivity when conditions are favourable. Moreover, captive males and females of some species do not mature and reproduce synchronously.[28,29] With intensification of aquaculture, interest in controlling crustacean reproduction using assisted breeding techniques, such as artificial fertilisation, has gained impetus.[25,28] Artificial fertilisation involves the manual collection and handling of spermatozoa (in a way that maintains their quality) in order to fertilise eggs *in vivo* or *in vitro* with maximum efficiency.[28] Gametes can be collected from superior broodstock with high-quality phenotypic traits and used in artificial fertilisation during a precisely controlled time window as compared to natural mating that could take several hours to days.[28] By so doing, the genetic selection for high-quality offspring can also be maximised, especially in commercial production.

In reproduction, the contribution of intact sperm DNA to the embryo is critical to ensure healthy development of offspring.[30–32] Until recently, male fertility has been assessed microscopically using traditional sperm quality parameters, such as sperm morphology and sperm concentration (using a counting chamber). However, these methods can be time-consuming and can only evaluate a limited number of sperm cells and males.[24] Even spermatozoa in high concentration that look morphologically normal according to traditional assessment methods, may not be capable of fertilising eggs.[30,31,33–35] As such, successful reproduction also entails high fertilisation competence, which can be more precisely evaluated through assessment of intracellular organelle functions, such as sperm plasma membrane integrity, mitochondrial function, acrosome reaction and DNA fragmentation.

Given that the fertility of male decapod crustaceans has received little attention, to date, no comprehensive review currently exists that specifically focusses on the evaluation, cryopreservation and artificial fertilisation of spermatozoa for these highly important aquacultural species. A recent review by Beirão et al. (2019) included a broad range of aquatic species such as finfish, bivalve molluscs, marine mammals and only penaeid shrimp represented decapod crustaceans.[28] Moreover, that review only focussed on current techniques for sperm collection, storage and artificial insemination.[28] This review hence attempts to fill the gap by focusing on recent advances for evaluating sperm quality to determine male fertility that could potentially be applied to decapod crustaceans, including those advanced techniques developed for mammals, as well as the current state of sperm cryopreservation and artificial fertilisation techniques and their implications for crustacean aquaculture. The development and optimisation of such fertility and assisted reproductive techniques may improve productivity in the crustacean aquaculture industry by early diagnosis of infertility and acceleration of selective breeding.

2 | COLLECTION AND EVALUATION OF SPERM QUALITY IN DECAPOD CRUSTACEANS

In general, the male reproductive system of crustaceans consists of testes and vas deferens (VD) that connect to external openings called gonophores; an elevated genital papillae in some species or an extruding copulatory structure in others.[36,37] Spermatogenesis in the decapod testis begins with the proliferation of spermatogonia and subsequent meiosis yielding primary then secondary spermatocytes

that eventually differentiate into spermatids. Through the process of spermiogenesis, spermatids develop into mature spermatozoa that are then transported to the vas deferens where they are gradually coated with 1–3 spermatophore layers during transit.[37,38] The spermatophore itself is a complex structure comprised of sperm-filled tubes coated with layers of a protective gelatinous matrix.[39] During copulation, spermatophores are extruded by the male through the paired gonophores located at the base of the walking legs and deposited inside the sex organ or attached to the ventral surface of the female, which may store it for a prolonged period prior to fertilisation.[28,39]

As such, in order to obtain spermatozoa to evaluate their quality, extraction of a spermatophore from the male reproductive tract is necessary.[28] Most knowledge in this regard is derived from economically important decapod crustaceans targeted for aquaculture. Not surprisingly, however, current sperm handling protocols vary greatly from one species to another.[28]

2.1 | Spermatophore extraction and semen extenders

Commonly, spermatophore extraction involves anaesthetising adult males at 10°C for 15–20 min and weighing them before using one of the following three methods to collect spermatophores. The first and most commonly adopted method involves post-mortem dissection of the reproductive tract, particularly the vas deferens, to expose the spermatophore; whose size, colour and consistency are examined macroscopically. For freshwater crayfish, often at least a 1 cm section of the distal vas deferens (DVD) is cut, and then placed in 1 mL of physiological saline solution, especially formulated for crustaceans.[15,16] This is similar in size transmitted to the female during copulation in freshwater crayfish.[15,16,20,21] The two other methods of spermatophore collection, that is, manual extrusion and electroejaculation, are non-lethal, hence have the benefit of avoiding killing valuable male broodstock.[28,40] Manual extrusion, commonly used in sexually mature male penaeids, can be performed by applying gentle pressure with the thumb and index finger laterally around the coxas of the fifth pair of walking legs of the male.[28,40] Frequent manual extrusion may cause inflammation of genitals and deposition of melanin (melanisation); triggered by the presence of haemocytes embedded around the connective tissue of the genitals.[41] Moreover, the reproductive tract can be damaged by melanisation of gonophores and sperm quality reduced if manual extrusion is performed incorrectly or too frequently.[41]

Electroejaculation can be an alternative method to stimulate extrusion of spermatophores from the gonophore of mature male decapods, including freshwater crayfish, freshwater prawn and lobsters. Electroejaculation is done by placing two electrodes at the base of the sternal keel near the coxa of the fifth walking legs of the male.[42–44] In the freshwater crayfish, *Cherax destructor*, an AC variable transformer delivered a maximum 55 V stimulus through a pair of electrodes at maximum of 10 s pulses between 40 and 60 Hz to the male to induce muscle contractions, which led to discharge of spermatophores.[40,42] While manual extrusion may be a more straightforward procedure, its success depends on the experience and skill of the handler as well as species. For example, manual extrusion is generally ineffective on hard-shelled crustaceans, such as freshwater crayfish and marine lobsters, for which electroejaculation may be the only non-lethal alternative.[4,42,43,45] By contrast, the intensity of electrical stimulation can be precisely controlled for electroejaculation, often leading to more consistent results.[4,42,43,45] The procedure of electroejaculation is believed to cause relatively limited discomfort to animals as long as low currents are used. Moreover, it is also considered safe and repeatable provided that animals are handled carefully and allowed sufficient time to recover between extractions.[41,44] Further methods include manual removal of spermatophores from the spermatheca, genitals or sternum of copulated female crustaceans using tweezers,[7,46–49] and Table 1 summarises these various methods of spermatophore extraction reported in literature.

Preparation of a single-cell suspension of spermatozoa is often a pre-requisite for sperm quality evaluation. In decapod crustaceans, a single-cell suspension of spermatozoa can be achieved mechanically by gentle homogenisation of the spermatophore using a tissue grinder, or by vigorous repeated pipetting in a semen extender solution to disrupt the walls of the spermatophore.[15,50–52] Extracted sperm cells are then filtered to remove any debris and pelleted by centrifugation at 200–500× *g* for 5 min. Spermatozoa are able to survive a series of resuspension and centrifugation steps during the washing process.[50] Spermatozoa can also be extracted from the spermatophore by chemical treatments such as trypsin[53,54] or pronase digestion[55] at 4°C for a period of time prior to mechanical homogenisation in semen extender. However, one must be careful with such treatments since pronase is known to induce acrosome reaction in spermatozoa, which should be avoided to prolong short-term sperm storage.[55]

Once spermatophores are extruded, they are often held in a semen extender solution prior to sperm quality assessment.[10,56] Semen extender is typically a physiological saline solution made of salts and sugars, which is added to seminal fluid to prolong sperm viability after collection of extruded spermatozoa.[10,57] One critical factor for a good semen extender is its ability to prevent functional activation during collection, handling and storage of spermatozoa.[26,57,58] It should also provide an isotonic environment for spermatozoa with good pH buffering capacity, and include nutrients and sugars necessary for sperm cell survival, as well as antioxidants to control reactive oxygen species and antibacterial substances to fight bacterial proliferation.[58,59]

While long-term cryopreservation of spermatozoa is discussed further in Section 3, short-term storage of both spermatophore or spermatozoa help enhance reproductive management at the hatchery by allowing more time to complete sperm quality assessments, and adding more flexibility to carry out breeding programmes, specifically for artificial insemination,[7,8,60] where spermatozoa need to be maintained *in vitro* for a short period prior to the insemination

TABLE 1 Methods of spermatophore extraction, handling, semen extenders and sperm quality evaluation reported for decapod crustaceans

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Freshwater crab						
Somanniathelphusa pax.; *Geothelphusa dehaani*	Post-mortem dissection	Testis	Fixed in Davidson's solution	Gonadosomatic index (GSI)	Tissue weight	[237]
Eriocheir sinensis	Post-mortem dissection	Spermatophore	Precooled (4°C) Ca^{2+}- free artificial seawater (FASW)	Cryopreservation-induced acrosome reaction (AR)	Light microscopy	[96]
Eriocheir sinensis	Post-mortem dissection	Spermatophore, sperm	Ca^{2+}- (FASW)	Acrosin activity, spermatophore digestion using accessory sex gland proteins	Scanning & transmission electron microscopy (SEM & TEM)	[238]
Eriocheir sinensis	Post-mortem dissection	Sperm	Ca^{2+}- FASW	AR	*In vitro* AR assay	[109]
Sinopotamon henanense	Post-mortem dissection	Testis	1:9 (w/v) 0.9% saline solution at 4°C.	Oxidative stress & apoptotic changes in testes against cadmium toxicity	Haematoxylin & eosin (H&E) staining, acridine orange (AO) / ethidium bromide (EB) dual fluorescent staining, TEM & DNA fragmentation analysis	[159]
Sinopotamon henanense	Post-mortem dissection	Seminal vesicle	Phosphate buffer saline (PBS; pH = 7.4)	Oxidative damage & ultrastructural changes after cadmium exposure	TEM; Malondialdehyde, MDA), proteins (protein carbonyl derivates, PCO) & DNA (DNA–protein crosslinks, DPC) biomarkers for oxidative damage	[158]
Barytelphusa Guerini	Post-mortem dissection	Testis	Ice-cold PBS	Morphology & GSI	Tissue weight; light microscopy	[239]
Sinopotamon henanense	Post-mortem dissection	Sperm	PBS	Sperm count, oxidative damage, sperm plasma membrane, AR, DNA integrity	Flow cytometry, fluorescent microscopy, reactive oxygen species (ROS), total antioxidant capacity (T-AOC), lipid peroxidation (MDA)	[95]
Freshwater crayfish						
Cambaroides japonicas	Post-mortem dissection	Testis	5°C in 1% OsO_4 (pH 7.2), veronal acetate for EM	Nuclear & cytoplasmic differentiation in sperm (sperm morphology)	TEM	[240]

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Cambaroides japonicus	Post-mortem dissection	Testis	4% buffered formaldehyde (pH 7.4), 1% OsO_4 (pH 7.4) for EM	Microtubular structure & sites of thiamine pyrophosphatase activity in premature sperm (sperm morphology)	Light microscopy & TEM	[70]
Cambarus sp.	Post-mortem dissection	Testis, vas deferens	Sodium eaeodylate buffered 5% glutaraldehyde (pH 7.3), 2% OsO_4 for EM	Acrosome formation, transformation of mitochondria & development of microtubules in sperm (sperm morphology)	TEM	[71]
Astacus astacus	Post-mortem dissection	Vas deferens	Fixed in 2.5% glutaraldehyde in Sorensen's buffer solution (pH 7.2)	Sperm morphology	TEM	[241]
Cherax Tenuimanus; *Cherax albidus*	Post-mortem dissection; Electroejaculation	Sperm	Not mentioned	Sperm morphology	Light microscopy & TEM	[69]
Cherax albidus	Post-mortem dissection	Vas deferens	Fixed in Bouin's solution	Spermatophore formation	Light microscopy & TEM	[242]
Orconectes propincus; *Janus frontalis*	Post-mortem dissection	Sperm	0.5% glutaraldehyde & 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.6) for EM; Hoechst 33258 for DNA localisation	Microfilament DNA localisation	Fluorescent microscopy; Laser scanning confocal microscopy (LSCM); SEM	[243]
Procambus paeninsulanus	Post-mortem dissection	Testis	Paraformaldehyde-glutaraldehyde fixative in 0.1 M phosphate buffer pH 7.2	Sperm morphology & spermatogenesis	Light microscopy & TEM	[244]
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens	Fixed in Bouin's solution	Male reproductive tract morphology	Tissue weight & light microscopy	[245]
Cherax destructor	Electroejaculation	Spermatophore	Not mentioned	Spermatophore morphology, ejaculation efficiency	No. of males extruding spermatophores from both gonophores	[42]
Cherax quadricarinatus	Post-mortem dissection	Androgen gland (AG), testis, vas deferens	Fixed in Bouin's solution	AG, testis & vas deferens weights, AG polypeptides	Tissue weight; histology; SDS-PAGE	[246]
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens spermatophore	Fixed in Bouin's solution	Male reproductive tract morphology	Histology	[247]

(Continues)

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Cherax quadricarinatus	Post-mortem dissection	Spermatophore	Fixed in Bouin's solution	Structural changes in spermatophore	Histology	[64]
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens, spermatophore, sperm	Physiological salt solution; Fixed in Bouin's solution	Sperm count & mortality, reproductive tract weight & morphology	Tissue weight; vital dye exclusion (10% methylene blue); histology	[15]
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens spermatophore, sperm	Physiological salt solution; Fixed in Bouin's solution	Sperm count & mortality, reproductive tract weight & morphology	Vital dye exclusion (10% methylene blue); histology	[16]
Astacus leptodactylus	Post-mortem dissection	Testis, vas deferens	Fixed in Bouin's solution	Morphology	Light microscopy	[248]
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens	Fixed in Bouin's solution	GSI, male reproductive tract morphology	Tissue weight & histology	[249]
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens	Fixed in Bouin's & Carnoy's solutions	Male reproductive tract morphology	Feulgen staining; light microscopy; TEM	[38]
Cherax quadricarinatus	Post-mortem dissection	Testis, vas deferens	Not mentioned	Male reproductive tract morphology	Light microscopy	[250]
Cherax quadricarinatus	Post-mortem dissection	Testis	Liquid nitrogen	Heat shock protein 70 influence on spermatogenesis	Real-time quantitative PCR	[251]
Austropotamobius italicus	Post-mortem dissection	Spermatophore	0.9% physiological saline	Sperm viability & longevity	Phase-contrast microscopy; Fluorescence microscopy	[252]
Astacus leptodactylus	Post-mortem dissection	Whole male reproductive tract	Physiological solution for freshwater crustaceans	Sperm number, male reproductive tract weight, testis & vas deferens weights, GSI, testicular index	Tissue weight & light microscopy	[21]
Astacus leptodactylus	Post-mortem dissection	Spermatophore, sperm	Not mentioned	Sperm number, reproductive tract morphology, GSI & testicular index (TI)	Spermatophore weight & light microscopy	[20]
Cherax quadricarinatus	Post-mortem dissection	Testis	Liquid nitrogen (LN_2)	Prohibitin characterisation	Gene expression; western blot analysis; immunofluorescent microscopy	[253]
Orconectes sp.; *Procambarus* sp.; *Astacus* sp.	Post-mortem dissection; Electroejaculation	Sperm	2.5% glutaraldehyde in 0.1 M phosphate buffer	Sperm ultrastructure	TEM	[72]

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Astacus leptodactylus	Electroejaculation	Spermatophore	Not mentioned	Post-mating spermatophore morphology	SEM & TEM	[218]
Pacifastacus leniusculus	Electroejaculation	Spermatophore	LN_2	Proteomic profiling	Two-dimensional electrophoresis & Western blotting	[80]
Cherax quadricarinatus; *Cherax destructor*	Electroejaculation	Spermatophore	2.5% glutaraldehyde in 0.1 M phosphate buffer	Comparative sperm ultrastructure	TEM	[68]
Astacus astacus	Electroejaculation	Spermatophore	LN_2	Protein profiling	In-gel trypsin digestion & high-resolution mass spectrometry	[225]
Pacifastacus leniusculus	Electroejaculation	Spermatophore	LN_2	Post-mating protein profile & pattern of protein tyrosine phosphorylation	Two-dimensional electrophoresis & Western blotting	[222]
Cambarus robustus; *Orconectes propinquus*; *Orconectes rusticus*	Post-mortem dissection	Sperm	2.5% glutaraldehyde in 0.1 M phosphate buffer	Sperm ultrastructure	TEM	[254]
Procambarus clarkii	Post-mortem dissection	Testis	Not mentioned	Kinesin-14 motor protein KIFC1function during spermatogenesis	Rapid-Amplification of cDNA Ends (RACE), *In situ* hybridisation	[255]
Pontastacus leptodactylus	Post-mortem dissection	Whole reproductive tract, vas deferens, testis	0.9% NaCl solution	Sperm count, GSI, testicular index, vas deferens index	Tissue weight & light microscopy	[256]
Pontastacus leptodactylus	Post-mortem dissection	Distal ductus deferens	0.9% NaCl solution	Sperm count, incubation time & temperature for extraction	Light microscopy	[19]
Pontastacus leptodactylus	Post-mortem dissection	Vas deferens, testis	Ca^{2+}- free saline	GSI, vas deferens index, testicular index	Tissue weights; haemocytometer counts; light microscopy	[17]
Pontastacus leptodactylus	Electroejaculation	Spermatophore	Tris buffer solution	Artificial extrusion, artificial insemination, sperm count	Eosin–nigrosin staining; light microscopy	[257]
Pontastacus leptodactylus	Post-mortem dissection	Whole reproductive tract	Not mentioned	Reproductive tract weight, testis weight, vas deferens weight, GSI, testicular index, vas deferens index, sperm count	Eosin–nigrosin staining; light microscopy; tissue weights	[18]

(Continues)

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Freshwater prawn						
Macrobrachium rosenbergii	Electroejaculation with 24h interval, gentle pressure on terminal ampullae post-mortem	Spermatophore	Buffered seawater (pH 7.4)	Artificial fertilisation, fertilisation rate	Fluorescent microscopy & SEM	[5]
Macrobrachium rosenbergii	Manual removal from female's sternum	Spermatophore	Ringer solution at 2°C or room temperature	Artificial insemination & short – term preservation	Fertilisation & hatching rates	[7
Macrobrachium rosenbergii	Manual removal from female's sternum	Spermatophore	10% glycerol in freshwater or physiological saline (pH 7.6)	Spermatophore cryopreservation	Fertilisation & hatching rates; post-thaw sperm fertilisation rate	[6]
Macrobrachium rosenbergii	Electroejaculation at different time intervals	Spermatophore	4% glutaraldehyde in 0.1 M Cacodylate-buffer	Sperm counts & morphology	Light microscopy & TEM	[44]
Macrobrachium malcolmsonii	Electroejaculation	Spermatophore	Ca^{2+}- free saline	Spermatophore weight, sperm count, % live & abnormal sperm	Spermatophore weight; Trypan blue staining & light microscopy	[258]
Macrobrachium rosenbergii	Electroejaculation	Spermatophore	Glycerol & ethylene glycol in deionising water	Sperm cryopreservation; artificial insemination	Trypan blue staining & fertilisation rate; light microscopy	[203]
Macrobrachium rosenbergii	Post-mortem dissection	Testis, vas deferens	Fixed in Davidson's solution	Spermatogenesis in testis	Light microscopy; SEM; TEM	[135]
Macrobrachium rosenbergii	Post-mortem dissection	Androgenic gland	Crustacean physiological saline	Testicular maturation & male sex development factors	Histology; BrdU proliferative cell assay; Immunofluorescent microscopy; ELISA; tissue weight	[259]
Macrobrachium rosenbergii	Electroejaculation	Sperm	Sterile-filtered pond water	Sperm cryopreservation	Eosin–nigrosin staining; light microscopy; phase-contrast microscopy; SEM	[202]
Macrobrachium acanthurus	Electroejaculation	Spermatophore	Distilled water	Sperm count, sperm cryopreservation	Eosin-nigrosin staining & light microscopy	[196]
Macrobrachium americanum	Electroejaculation	Spermatophore, sperm	Distilled water	Sperm count, sperm viability & normal/abnormal morphology	Eosin-nigrosin staining; Light microscopy; proximate analysis of spermatophores; histology	[260]

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Marine crab						
Scylla serrata	Post-mortem dissection	Spermatophore & seminal plasma	0.1 M glycine in phosphate buffer saline	Morphology & cryopreservation of spermatophores, sperm viability assay	Eosin-nigrosin staining	[205]
Scylla serrata	Post-mortem dissection	Spermatophore, sperm	Calcium ionophore A23187 for AR; Ca^{2+}- FASW	Viability of cryopreserved sperm	Artificial induction of AR; hypo/hyperosmotic sensitivity tests for membrane integrity; trypan blue & eosin-nigrosin staining; light microscopy	[55]
Uca pugilator	Post-mortem dissection	Testis	Fixed in Bouin's solution	Testicular maturation index using 5-Hydroxytryptamine (5-HT)	5-HT injection on D1, 5 & 10, then crabs sacrificed on D15.	[261]
Portunus trituberculatus	Post-mortem dissection	Sperm	Ca^{2+}- FASW	AR, sperm cryopreservation	Calcium ionophore A23187 treatment	[262]
Birgus latro	Post-mortem dissection	Testis, vas deferens	120 min in 20% NaOH	Sperm count	Stereomicroscopy	[263]
Perisesarma bidens	Manual removal from the spermatheca	Sperm	Filtered seawater	Artificial insemination	Fertilisation rate	[47]
Scylla serrata	Manual removal from the spermatheca	Spermatophore	Ca^{2+}- FASW	AR	Light microscopy; calcium ionophore A23187 to induce AR *in vitro* assay; TEM	[48]
Charybdis japonica	Post-mortem dissection	Sperm	15% DMSO	Sperm viability, sperm cryopreservation	Light microscopy	[264]
Portunus pelagicus	Post-mortem dissection	Testis, vas deferens	Davidson's fixative; 4% glutaraldehyde & 2% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C.	Spermatogenesis (morphology)	Light microscopy & TEM	[265]
Cancer setosus	Post-mortem dissection	Spermatophore	Filtered seawater	Structural & ultrastructural events during the acrosome reaction	Light microscopy & SEM	[102]
Callinectes danae	Post-mortem dissection	Spermatophore, sperm	4% paraformaldehyde	GSI	Histochemistry & light microscopy	[266]

(Continues)

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Callinectes ornatus	Post-mortem dissection	Testis, vas deferens	4% paraformaldehyde in saltwater	Male reproductive tract histology & histochemistry; GSI	Light microscopy & gonadal weight	[267]
Scylla olivacea	Post-mortem dissection	Sperm	Ringer solution	AR	Eosin-nigrosin staining & light microscopy	[101]
Portunus armatus	Post-mortem dissection	Whole male reproductive tract	Fixed in Bouin's solution	Male reproductive tract morphology	Light microscopy & TEM	[268]
Charybdis japonica	Manual removal from the spermatheca	Sperm	Ca^{2+}- free saline	Sperm viability, acrosin activity, sperm cryopreservation	Nα-benzol-DL-arginine-p-nitroanilide (BAPNA) substrate method for acrosin activity, eosin B-staining, microscopy; sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)	[46]
Scylla tranquebarica	Post-mortem dissection	Sperm	Ca^{2+}- free saline	Sperm count & viability, sperm cryopreservation	Light microscopy	[207]
Marine lobster						
Homarus americanus	Post-mortem dissection	Testes, vas deferens	Seawater, calcium ionophore A23187 plus 1% DMSO in seawater	AR	Phase-contrast microscopy	[97]
Homarus americanus	Electroejaculation (12V), spermatophore cut to obtain sperm	Spermatophore	100 μmol L^{-1} calcium ionophore A23187	Sperm morphology, AR, vas deferens morphology after extrusion	Light microscopy & TEM	[45]
Homarus americanus	Electroejaculation (10mA; 7–8 V; 300–2500 ohms)	Spermatophore	Seawater	Spermatophore quality (presence of sperm), artificial insemination & fertilisation	No. of spermatophores, sperm count & fertilisation rate	[43]
Homarus americanus	Electroejaculation, spermatophores cut in seawater & sperm mass extruded to form suspension	Spermatophore	Seawater, paraffin oil (Fisher, Saybolt viscosity 125/135), 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for EM	Morphology & cryopreservation of spermatophores	Light microscopy & TEM	[199]

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Panulirus homarus	Post-mortem dissection	Testis, vas deferens	Davidson's fixative	Male reproductive tract histology & ultrastructure	Light microscopy & TEM	[269]
Panulirus polyphagus	Post-mortem dissection	Sperm	Ca^{2+}- free saline	Sperm counts & viability, sperm cryopreservation	Eosin-nigrosin staining & light microscopy	[206]
Marine shrimp						
Penaeus aztecus	Post-mortem dissection	Testes, vas deferens	Karnovsky's fixative	Sperm morphology	Phase-contrast microscopy & TEM	[270]
Penaeus setiferus; *P. stylirostris*; *P. vannamei*	Electroejaculation	Spermatophore	Direct to female's sternum	Artificial insemination using α-cyanoacrylate adhesive	No. of spermatophores expelled	[4]
Penaeus setiferus	Electroejaculation	Sperm	Sperm released from sperm mass by glass tissue grinder into Ca^{2+} - free saline (pH 7.4)	Spermatophore weight, sperm count, % live sperm, % abnormal sperm trypan blue staining	Light microscopy	[52]
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+}- FASW	Sperm count, morphology & sperm viability	Spermatophore weight; light microscopy for morphology & count; trypan blue staining; acridine orange fluorescent assays & microscopy in sperm viability; EW- induced sperm reaction	[103
Litopenaeus vannamei	Manual extrusion	Spermatophore	Seawater or Ca^{2+}- free saline	Short-term storage (36h)	Sperm count & morphology using phase-contrast microscopy	[271]
Penaeus setiferus	Electroejaculation with antibiotic after extrusion	Sperm	Ca^{2+}- free saline	Sperm morphology, GSI, sperm count	Trypan blue staining & light microscopy	[272]
Penaeus indicus	Electroejaculation	Spermatophore	Filtered seawater	Cryopreservation of spermatozoa	EW-induced acrosome reaction	[273]
Metapenaeus affinis; *Metapenaeus brevicornis*	Electroejaculation	Sperm	Not mentioned	Artificial insemination with & without α-cyanoacrylate	No. of spermatophore expelled; spawning & hatching rates	[274]
Pleoticus muelleri	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Spermatophore weight, sperm count, sperm viability	spermatophore weight; trypan blue staining & light microscopy	[275]
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Sperm count & viability	Light microscopy	[276]

(Continues)

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Trachypenaeus byrdi; *Xiphopenaeus riveti*; *Litopenaeus occidentalis*	Manual extrusion	Spermatophore	Filtered natural seawater	AR	EW technique &SEM	[224]
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Spermatophore weight, sperm count, sperm viability	Spermatophore weight; trypan blue staining; light microscopy	[277]
Litopenaeus vannamei	Manual extrusion	Spermatophore, sperm	Sterile seawater	Sperm, spermatic mass & whole spermatophore cryopreservation	Light microscopy; flow cytometry (DNA staining with propidium iodine; PI)	[24]
Farfantepenaeus paulensis	Manual extrusion	Spermatophore, sperm	Not mentioned	Artificial insemination	Fertilisation rate	[278]
Farfantepenaeus paulensis	Manual extrusion	Spermatophore, sperm	Ca^{2+}- free saline	Spermatophore weight, sperm count	Tissue weights & light microscopy	[279]
Penaeus monodon	Manual extrusion	Spermatophore	Mineral oil (0.1% penicillin-streptomycin)	Sperm viability, artificial insemination, chilled storage of spermatophore	Eosin–nigrosin staining & light microscopy	[198]
Penaeus monodon	Manual extrusion	Spermatophore, sperm	5% DMSO + Ca^{2+}- free saline (0.09% NaCl)	Cryopreservation & artificial insemination, sperm count, sperm viability, spermatophore weight, abnormal sperm	Tissue weights; fertilisation rate; light microscopy	[8]
Litopenaeus vannamei	Manual extrusion	Spermatophore	Mineral oil with 0.1% penicillin-streptomycin	Sperm viability, chilled storage of sperm	Eosin–nigrosin staining & light microscopy	[197]
Penaeus monodon	Manual removal from female's thelycum	Spermatophore, sperm	Artificial filtered seawater	Acrosome reaction	EW technique; SEM; TEM	[49]
Penaeus monodon	Manual extrusion	Spermatophore, sperm	Ca^{2+}- free saline	Sperm viability, spermatophore cryopreservation	Eosin-nigrosin staining; light microscopy; fertilisation rate	[9]
Farfantepenaeus paulensis	Manual extrusion; electroejaculation	Spermatophore	Ca^{2+}- free saline	Sperm count; spermatophore weight; spermatosomatic index (ESI)	Spermatophore weight & light microscopy	[280]
Penaeus monodon	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Sperm viability, bacterial profiling for 210 d	Eosin–nigrosin staining; light microscopy; bacterial assay (incl. aseptic techniques)	[204]

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Litopenaeus vannamei	Manual extrusion	Spermatophore	Trypsin at 4°C for 24 h, Ca^{2+}- free saline at 25°C	Sperm count, sperm cryopreservation	Flow cytometry; eosin-nigrosin staining; light microscopy	[54]
Farfantepenaeus paulensis	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Spermatophore weight, sperm count, melanisation & spermatophore absence rates	Spermatophore weight & light microscopy	[281]
Litopenaeus vannamei	Manual extrusion	Spermatophore	2.5% glutaraldehyde in artificial seawater (pH 8.0); 4% paraformaldehyde	Ultrastructure & biochemistry of sperm capacitation	EM/LSCM; confocal immunofluorescent microscopy to detect tyrosine-phosphorylated proteins	[223]
Rhynchocinetes typus	Post-mortem dissection; electroejaculation	Vas deferens	Filtered seawater	Gamete & zygote morphology during fertilisation	Light microscopy; confocal fluorescent microscopy; SEM; TEM	[282]
Penaeus merguiensis	Manual extrusion	Spermatophore	Ca^{2+}- free saline	Sperm viability, spermatophore cryopreservation	Eosin-nigrosin staining; light microscopy; fertilisation rate	[201]
Litopenaeus vannamei	Manual extrusion	Sperm	Modified artificial saline (antibiotic/antimycotic, pH 7.4)	Short-term storage of sperm, artificial insemination	Fertilisation rate	[10]
Litopenaeus vannamei	Manual extrusion	Spermatophore, sperm	Ca^{2+}- free saline	GSI, spermatophore energy levels & sperm count	Trypan blue exclusion test; light microscopy; sex organ weight	[283]
Stenopus hispidus	Post-mortem dissection	Whole reproductive tract	2.5% glutaraldehyde & 4% paraformaldehyde in 0.1 mol L^{-1} phosphate buffer (pH 7.3; Karnovsky solution)	Male reproductive tract morphology	SEM	[284]
Litopenaeus schmitti	Post-mortem dissection	Spermatophore	Ca^{2+}- free saline	Sperm cryopreservation, sperm count	Eosin-nigrosin staining & light microscopy	[285]
Farfantepenaeus paulensis	Manual extrusion	Sperm	Ringer solution	Sperm count, DNA damage	Eosin-nigrosin staining, SEM; Comet assay	[286]
Litopenaeus vannamei	Manual extrusion	Spermatophore	Sterile seawater; Freezing solution (5% egg yolk & 0.2 M sucrose)	Sperm count, sperm cryopreservation	Eosin-nigrosin staining; light microscopy; flow cytometry	[94]

(Continues)

TABLE 1 (Continued)

Species	Method of extraction	Reproductive tissues/cells	Semen extender/fixative	Fertility biomarkers	Techniques for assessment	References
Litopenaeus vannamei	Manual extrusion	Sperm	Ca^{2+}- free saline	Sperm viability, sperm cryopreservation	Eosin–nigrosin staining; 6-carboxyfluorescein diacetate (CFDA) & PI staining; fluorescent microscopy	[195]
Litopenaeus vannamei; *Farfantepenaeus*; *subtili*; *Litopenaeus schmitti*	Manual extrusion	Sperm	Ca^{2+}- FASW	Spermatophore weight, sperm count & sperm viability	Light microscopy; fluorescence microscopy	[93]
Penaeus monodon	Post-mortem dissection	Testis	Davison's solution; 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 mol L^{-1} sodium cacodylate with 5% sucrose (pH 7.4)	Spermatogenesis (morphology)	Light microscopy & TEM	[129]
Litopenaeus vannamei	Manual extrusion	Spermatophore	LN_2 for vitrification Ca^{2+}- free saline & 0.4 M trehalose	Sperm vitrification, sperm count, artificial insemination, membrane integrity	Fluorescent microscopy & light microscopy	[34]
Palaemon serratus	Manual extrusion	Sperm	Artificial seawater	DNA integrity	Comet Assay	[142]
Penaeus monodon	Post-mortem dissection	Reproductive organ	Davidson's solution	Acrosome formation & spermatophore formation	Histology; light microscopy; TEM	[287]
Penaeus monodon	Electroejaculation	Spermatophore	Ca^{2+}- free saline	DNA fragmentation	Comet assay, sperm chromatin dispersion test (SCDt)	[157]
Fenneropenaeus indicus	Manual extrusion	Sperm	Ca^{2+}- free saline	Sperm viability, sperm cryopreservation	Eosin-nigrosin staining; light microscopy; Hypo-osmotic swelling test (HOST) & DNA integrity analyses; fluorescent microscopy.	[81]
Penaeus monodon	Electroejaculation	Spermatophore	Artificial lobster haemolymph (AH), Ca^{2+}- free saline, & Ca^{2+}- FASW	Plasma membrane integrity, % AR, & % sperm DNA fragmentation (SDF) after 4°C storage for 0–26 d	AR assay *in vitro*; Sperm viability assay; Fluorescent microscopy	[51]

procedure.[10] By developing an optimised semen extender, the number of females inseminated per male can be maximised, and time window to execute artificial insemination can be increased.

In *Penaeus* shrimp, a species-specific artificial semen extender solution was developed for whiteleg shrimp consisting of 2.125 g L^{-1} NaCl, 0.110 g L^{-1} KCl, 0.052 g L^{-1} H_3BO_3, 0.019 g L^{-1} NaOH and 0.484 g L^{-1} $MgSO_4 \cdot 7H_2O$ with 20 μL of antibiotic/antimycotic (10,000 U Penicillin, 10 mg Streptomycin, 25 μg Amphotericin B) at pH 7.4. This extender reportedly facilitated >60% fertilisation rate following artificial insemination.[10] Whiteleg shrimp spermatophores stored in this extender can be maintained for up to 26 h at 14°C with 92 ± 15% spermatozoa showing normal morphology.[61] For decapods, Ca^{2+}- free saline is normally recommended as a semen extender during sperm cryopreservation since the absence of calcium prevents the initiation of the acrosome reaction in spermatozoa.[9,51,62] Ca^{2+}- free saline is composed of 21.63 g L^{-1} NaCl, 1.12 g L^{-1} KCl, 0.53 g L^{-1} H_3BO_3, 0.19 g L^{-1} NaOH, 4.93 g L^{-1} $MgSO_4 \cdot 7H_20$ at pH 7.5.[9] A list of different semen extender solutions and sperm quality assessments for crustaceans is shown in Table 1, and they include physiological saline for freshwater crustaceans; filtered natural or artificial seawater, or mineral oil for marine crustaceans. Meanwhile, Ca^{2+}- free saline or Ringer's and phosphate buffered saline solutions have been used for both marine and freshwater crustaceans.

2.2 | Advanced tools for sperm quality evaluation

The ultimate measures of male fertility in crustaceans are fertilisation and hatching rates. However, these measures take time to properly confirm, require the simultaneous availability of eggs to conduct the tests, and can also be significantly affected by female factors such as egg/embryo quality and culture conditions, which may yield variable results. Thus, assessment of male fertility by direct evaluation of sperm quality is a more feasible alternative since it can rapidly and objectively detect deleterious effects caused by poor male health, nutrition, husbandry, genetics or other factors.[63]

2.2.1 | Sperm number, morphology and membrane integrity

Once the spermatophore is collected and is allowed to soften in physiological saline for crustaceans, a single-cell suspension of spermatozoa can be prepared by gentle agitation, which then permits sperm number to be quantified.[15,16,20,21,56,64] Generally, greater numbers of spermatozoa are associated with greater reproductive capacity among males of most species.[62,63,65–67] In freshwater crayfish, sperm number is determined using a Neubauer haemocytometer and is typically expressed as the number of sperm/DVD section. In male redclaw weighing 70–110 g, sperm concentration ranges from 10^8 to 10^9 sperm/1 cm DVD section.[15,16] Typically, decapod spermatozoa are non-motile and without flagella.[37,39,68,69] Their spermatozoa are generally composed of a main body enclosing a decondensed nucleus, a highly complex acrosome in the anterior region, and no discernible midpiece (compare to mammalian sperm). From the main body, different numbers of stellate processes that project outside arise, but almost in a species-specific manner.[22,37] Thus, assessing sperm quality using simple light microscopy is difficult because distinct morphological features are lacking.[24] Moreover, sperm motility, which is a good biomarker to assess competitive swimming ability to fertilise eggs during spawning in finfish and other species, cannot be used for these crustaceans.[24] As such, researchers tended to use higher magnification microscopy such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to study decapod sperm morphology,[38,68–72] which is time-consuming, costly, and limited to low numbers of cells and individuals that can be evaluated. Thus, the need to more rapidly assess decapod spermatozoa has stimulated the adoption of newer methods.[22,24]

Abnormal sperm morphology can also be an indicator of infertility or aberrant spermatogenesis.[13,52,73,74] In decapod crustaceans, live spermatozoa can be examined by light microscopy for sperm abnormalities such as deformed or missing heads, twisted or missing spikes, and distorted main bodies.[13,52,73] However, the absence of distinct morphological structures in mature decapod spermatozoa, such as a lack of flagella or few or absent mitochondria, makes the rapid assessment of normal *vs.* abnormal morphology by light microscopy more difficult.[24,68,69]

Given the limitations mentioned above, alternative approaches have been considered such as evaluating the integrity of the plasma membrane to assess sperm viability.[75–80] For sperm viability in decapod crustacean, spermatozoa are typically stained with conventional (Trypan blue or eosin-nigrosin) or fluorescent (propidium iodide, PI) exclusion dyes in Ca^{2+}- free saline (pH 7.4) to examine the integrity of the cell's plasma membrane to determine the proportion of live (unstained) vs. dead (stained) spermatozoa.[22,52,73,81–83] The presence of calcium in semen extenders can trigger the acrosome reaction.[84–86] Thus, Ca^{2+}- free saline is generally used as the semen extender in most sperm viability studies because the absence of calcium prevents the acrosome reaction and subsequent rupture of the sperm plasma membrane.[9,51,62] Cells treated with conventional stains require light microscopy to manually detect and count dead cells; which can be labour intensive, permitting typically only ~200 cells per individual to be examined.[24,52,87] By contrast, treating cells with structure-specific fluorescent stains, enables the rapid analysis of more than 20,000 cells per individual to be examined in a matter of minutes using high-throughput flow cytometry.[87–90]

When using flow cytometry, spermatozoa are often counterstained with carboxyfluorescein diacetate (CFDA), SYBR®-14, or Hoechst 33342.[88,91,92] CFDA binds to esterases, which stains live spermatozoa with intact cell membranes fluorescent green; while SYBR®-14 and Hoechst 33342 bind to nucleic acids, which stains the nucleus of live spermatozoa green and blue respectively.[92,93] By contrast, PI binds to nucleic acids but can only penetrate cells that have ruptured membranes, causing the nucleus of dead spermatozoa to stain fluorescent red.[91–93] In crustaceans, fluorescent dyes coupled with flow cytometry showed 33%–89% post-thaw viability in

whiteleg shrimp spermatozoa after exposure to different cryopreservation protocols.[24,54,94] In freshwater crabs, PI staining and flow cytometry showed that sperm plasma membranes were significantly damaged (17%–20% dead spermatozoa) when exposed to high concentrations of lead.[95] These results show that fluorescent stains coupled with flow cytometry represent a sensitive tool for assessing sperm cell viability in decapod crustaceans.

2.2.2 | Acrosome reaction

The acrosome reaction (AR) of spermatozoa involves the fusion of the outer acrosomal membrane with the overlying sperm plasma membrane causing a release of digestive enzymes that permits a spermatozoon to penetrate the outer membrane of the egg, and by so doing deliver paternal genetic material into the egg that is required for successful fertilisation.[96] AR in decapod spermatozoa involves molecular and morphological changes to the acrosomal vesicle, and the introduction of subacrosomal and nuclear materials into the egg.[97] AR is a reliable predictive biomarker of sperm quality especially for non-motile spermatozoa often found in decapod crustaceans.[96,98] However, infertile males can have damaged/absent acrosomes thereby preventing such spermatozoa from fertilising eggs. In addition, various external stressors during semen processing and/or freezing can induce damage to the acrosome membrane or can provoke a premature acrosome reaction, leading to misleading infertility diagnoses that need to be carefully controlled.[99,100]

AR has been well studied in decapod crustaceans including crabs, lobsters and Penaeid shrimp.[48,49,53,96,98,101,102] Briefly, the AR can be induced by suspending spermatozoa in physiological saline that has been used to incubate eggs *in vitro* (ie egg water; EW). Alternately, AR can be observed naturally at the time of oviposition of a copulated female, which involves immediate collection of eggs and spermatozoa from the female's seminal receptacle.[49]

For decapod sperm, the capability to undergo AR is considered as a good biomarker of normal acrosome functionality.[51,103] The AR in decapods can be induced in several ways, including exposure to egg water, calcium ionophore, alkalinisation, high concentrations of ions, and cold shock.[48,86,96,98,104] Egg water, which is commonly used for AR induction *in vitro*,[49,105] typically contains vitelline envelope (VE), the external layer of eggs, cortical rods (CRs; egg jelly material located in egg surface crypts), and some thelycal (T) substances.[49,105–107] Egg water has approximately a 4:1 protein to carbohydrate ratio and contains trypsin-like enzymes, which are natural inducers that play a crucial role during the second stage of AR in decapods.[105,106] Calcium ionophore A23187 can also induce the AR in decapod spermatozoa by increasing calcium influx into the cells.[86,107] In the freshwater Chinese mitten crab (*Eriocheir sinensis*), AR reportedly can be induced by egg water, seawater, $CaCl_2$ solution or low temperature.[108,109] In addition, cryopreservation can also trigger AR in spermatozoa of *E. sinensis*[96] by directly promoting membrane fusion of the acrosomal cap, or by destruction of AR inhibiting and activation of AR promoting proteins.[96,110]

Presently, efforts to quantify acrosome reaction in decapod crustaceans involve counting of acrosome-reacted vs. normal intact spermatozoa based on their morphological appearance using light microscopy.[51,96] However, this requires considerable familiarity with acrosome morphology and the mechanism of AR for the decapod species of interest.[49,96,97,111] Moreover, some decapods (infraorder Caridea) appear to lack acrosome-like structures precluding the evaluation of the AR in these species.[111] By contrast, acrosome-specific biostaining techniques using fluorescein isothiocyanate (FITC)-conjugated *Arachis hypogaea* (peanut) lectin (FITC-PNA) can be employed to analyse the acrosome reaction across multiple decapod groups. The PNA lectin is specific for terminal β-galactose moieties and so will bind to the acrosome in acrosome-reacted sperm, fluoresce green,[22,112] and can be quantified via fluorescent microscopy or flow cytometry without the need to understand species-specific acrosome morphologies.[113–115]

2.2.3 | Mitochondrial function

An array of fluorescent dyes can be used as biomarkers for other specific sperm functions. Mitochondria are the source of ATP production in most eukaryotic cells, which is the basic unit of energy used to power their function.[116] Several dyes have been employed to assess mitochondrial membrane integrity and functionality. For example, mitochondrial stain, MitoTracker-Red CMXRos (M-7512) has been used to stain functional mitochondria fluorescent red in viable cells with dye accumulation due to mitochondrial membrane potential.[22] However, the most common combination of dyes used in aquaculture species is dual staining using rhodamine 123 (R123) and PI. Spermatozoa with intact plasma membrane and functional mitochondria take up R123 and fluoresce green, while PI stains the nucleus of dead cells with damaged membranes fluorescent red.[88,117] In aquaculture, the R123/PI staining method coupled with flow cytometry has been used to evaluate mitochondrial function in both marine invertebrates[118] and finfish.[89,119,120] The mitochondrial stain carbocyanine fluorescent probe, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetrathylbenzimidazolyl-carbocyanine iodide (JC-1) is another widespread fluorochrome used to assessed changes in mitochondrial membrane potential in mammalian sperm.[121–123] JC-1 can distinguish between spermatozoa with high *versus* low functional mitochondria.[87] JC-1 stained spermatozoa fluoresce green when mitochondrial functional is low; fluoresce orange/green when mitochondrial function is high; and fluoresce red when mitochondrial function is extremely high.[87,124–126] The protonophore, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) impedes mitochondrial function by uncoupling oxidative phosphorylation, and has been employed as a positive control for various cell types stained with JC-1 including both marine invertebrates[126,127] and finfish[128] spermatozoa.

Presently, there are no reports documenting mitochondrial function in decapod crustaceans. This may be related to the observation that mitochondria in mature decapod spermatozoa appear to be degenerate, transformed, non-christate, non-functional or even

absent in some species.[37,71,111,129] In some decapods, such as freshwater crayfish, few mitochondria have been observed to be associated with the membrane lamellae complex.[68,69] Mitochondria in this taxonomic group do not generate energy, but Anderson and Ellis (1967) have reported that the membrane lamellae complex is responsible for ATP production in mature spermatozoa of crayfish.[71] In the Danube crayfish (*Astacus leptodactylus*), the membrane lamellae complex was also reported to separate from the cell after discharge of spermatozoa from the spermatophore.[80] Given spermatozoa of most species of decapods are immotile, and few studies have reported functional mitochondria, the role of sperm mitochondria in male fertility may be relatively limited compared to fish and mammalian counterparts. However, the above assays may still help facilitate a greater understanding of the function of the membrane lamellae complex as energy source and the role (if any) of mitochondria during fertilisation in mature spermatozoa of decapod crustaceans.

2.2.4 | Sperm DNA fragmentation

Sperm DNA plays a crucial role in facilitating normal embryo development and live birth.[130–132] As such, DNA is highly condensed and efficiently packed in spermatozoa to avoid damage during transport to the site of fertilisation.[131–133] Sperm DNA is wrapped around histone proteins, which during spermatogenesis, are gradually replaced by highly basic protamines that facilitate greater condensation.[131–133] During this process, transcription and translational of sperm DNA ceases. Moreover, during condensation, double-stranded DNA incurs torsional stress resulting in nicks and breaks along the DNA strand.[132,133] Failure to repair these nicks and breaks combined with the cumulative effect of reduced protamination could lead to DNA damage.[131–134]

Interestingly, decapod spermatozoa are composed of a main body that envelops a decondensed nucleus of chromatin fibres,[68,129,135] where histones are relatively low to nil and protamines are completely absent.[11,135–137] Spermatozoa with poor chromatin packaging and low protamine content are susceptible to oxidative stress (imbalance between oxidation and reduction reactions).[138] Such a decondensed nucleus, composed of diffuse and heterogeneous chromatin fibres in decapod crustacean spermatozoa, can be highly susceptible to DNA damage.[139,140] The reactive oxygen species (ROS) generated by oxidative stress are an intrinsic source of sperm DNA damage.[138,141] Oxidative stress is caused by insufficient antioxidants to neutralise free radicals generated during spermatogenesis. ROS attack spermatozoa during spermatogenesis by activating endonucleases or caspases that cause DNA damage. Caspases are enzymes directly involved in the DNA fragmentation and cell death process.[138] In contrast to egg cells, spermatozoa lack the ability to prevent and repair DNA damage induced by environmental stressors.[139,140,142] Oxidative stress can also result in lipid peroxidation, protein alterations, and sperm apoptosis, which further compromise the paternal DNA contributed to the developing embryo.[132,138,143,144] Increased DNA damage in decapod spermatozoa has been reported when exposed to pollutants, such as heavy metals,[95,140,145] and environmental stressors, including low temperature[146] and extremities in pH.[147]

Recently in vertebrates, supplementation of antioxidants either in the diet or semen extender reduces the effects of oxidative stress on sperm DNA integrity; mitigating the effect of ROS.[90,132,148–150] For example, the use of antioxidants such as α-tocopherol and ascorbic acid in sperm freezing medium reduced lipid peroxidation and increase fertilisation rate (80%–90%) in cryopreserved spermatozoa of Atlantic salmon.[148] Alternatively, antioxidant supplementation of boar diets resulted in a 55% reduction in sperm DNA damage induced by heat-stress.[90,149] To date, the use of antioxidants to improve sperm quality in decapod crustaceans, has not been reported, warranting further investigation.

Sperm DNA damage is considered as a crucial indicator of male infertility.[151] Spermatozoa with DNA damage may look healthy when using traditional measures of assessing sperm quality and can still fertilise oocytes.[30,31,33–35] However, the structural damage in DNA can lead to abnormalities in pronuclear formation, activation of key embryonic genes and early embryo development.[131,152] During embryo development, the first 2-cell divisions are primarily controlled by maternal reserves of proteins and enzymes accumulated in the egg, but these need to be replenished by activation of the embryonic genome (containing both maternal and paternal DNA) from around the 4-cell stage in most species.[153] High levels of sperm DNA damage can induce delayed embryo cleavage, abnormal embryo morphology, and lower rates of blastocyst formation and implantation.[130,154–156]

DNA damage has been reported to occur in the spermatozoa of both vertebrates and invertebrates, including crustaceans.[30,142,157] For example, in whiteleg shrimp, sperm DNA damage was thought to be the cause of low to zero hatching rates after artificial insemination, despite good rates of egg fertilisation.[34] Chinese freshwater crabs (*Sinopotamon henanense*) exposed to higher concentrations of various heavy metals exhibited poor sperm quality with a high proportion of DNA fragmentation.[95,158,159] Although conventional sperm analyses such as sperm counting and spermatophore weight can be used to characterise sperm quality, molecular assays such as sperm DNA fragmentation (SDF) analysis, can provide insight into the developmental competence of embryos fertilised by a male's sperm, and hence, a more accurate measure of fecundity.[90,131,149,151] Thus, development of sperm DNA damage assays for decapod crustaceans could identify putative causes of poor juvenile production yields in commercial crustacean aquaculture.

Several assays have been developed to directly or indirectly measure sperm DNA damage, including traditional staining methods, toluidine blue staining, sperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD) or Halosperm test, terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), and the Comet assay.[133] However, given their different mechanisms for detecting DNA damage, the results from each test are unique and not comparable.[90,131,160] Given that sperm DNA damage can increase significantly after prolonged storage or incubation during sample preparation,[161] spermatozoa should be fixed shortly after

collection and then either: (i) smeared, air-dried and stained on glass slides before analysis by light/fluorescent microscopy, or (ii) washed, permeabilised and stained before analysis by flow cytometry.[161]

Toluidine blue staining is used to evaluate damage to the nuclear chromatin structure of spermatozoa and is visualised by light microscopy.[133,162] Using toluidine blue, sperm heads with high chromatin integrity stain blue, while sperm heads with damaged chromatin stain purple.[162] By contrast, chromomysin A3 (CMA3) dye preferentially binds to spermatozoa with protamine deficiency, indicating that DNA is poorly packed or damaged.[163,164] As a result, faint yellow CMA3 dye staining indicates spermatozoa with normal or high protamination, whereas bright yellow staining indicates protamine deficiency associated with high DNA damage.[164,165]

The sperm chromatin structure assay (SCSA) is commonly used to detect sperm DNA damage in vertebrates.[87,133] It is an indirect assay in which DNA is denatured by heat or acid treatment causing single-stranded DNA breaks followed by AO staining.[166] AO binds to intact double-stranded DNA or denatured ssDNA to exhibit intense green or red fluorescence, respectively; which can be further assessed through a flow cytometer.[133,166] This assay can evaluate both fresh and frozen samples with high repeatability with less inter- and intra-sample variability.[133,134,167,168] However so far, there is no report on the use of SCSA in fish or crustaceans.[25]

Sperm chromatin dispersion (SCD) or Halosperm test is another indirect assay to evaluate sperm DNA damage. For SCD assay, spermatozoa are embedded in agarose on a slide and their DNA denatured with an acid solution to yield halos or dispersed chromatin due to relaxed DNA, which can then be visualised by fluorescent microscopy.[133] Spermatozoa with fragmented DNA produce small or no halos of dispersed DNA, while spermatozoa with intact DNA produce medium to large halos of DNA.[169–171] In the black tiger prawn, this assay found greater sperm DNA fragmentation in domestic vs. wild-caught individuals (6.8 ± 4.5% vs. 3.3 ± 1.5%; $n = 10$).[157] Thus, the SCD test could be a reliable predictive biomarker to assess male fertility for broodstock management in saltwater prawn aquaculture.[157]

The comet assay, an alkaline version of single-cell gel electrophoresis, is a test in which spermatozoa are embedded in agarose on a slide, lysed with detergent to release DNA, then subjected to electrophoresis before DNA is stained by SYBR Green I for visualisation using fluorescent microscopy.[157] During electrophoresis, small fragmented DNA strands migrate through the agarose away from the nucleus and are visualised as a 'comet-like' tail, while larger intact DNA remains compact in the sperm head. The length of migration of the tail and the intensity of fluorescent green staining is directly proportional to the amount of DNA damage within each spermatozoon.[172] While the comet assay can detect many types of DNA fragmentation, it is effective for fresh samples and requires only a few cells per analysis.[173,174] The Comet assay has been used to evaluate DNA fragmentation in crustacean spermatozoa. In black tiger prawn, a two-tail comet assay was performed to qualitatively validate sperm nuclear morphologies and % SDF determined using the SCD test.[157] Erraud et al. (2018) utilised the comet assay to determine declines in sperm quality among palaemonid prawns exposed to contamination; subsequently recommending its use as a potential predictive marker for *in situ* biomonitoring surveys.[142]

While the comet assay relies on fluorescent microscopy and only works for fresh samples, TUNEL assay can utilise either fluorescent microscopy or flow cytometry on fresh, fixed or cryopreserved samples.[134] TUNEL is a direct assay that targets DNA strand breaks by incorporating fluorescein isothiocyanate (FITC) conjugated 2′-deoxyuridine 5′-triphosphates (dUTPs) to the 3'hydroxyl (OH) breaks of single-stranded and double-stranded DNA. As such, the nucleus of DNA-damaged cells fluoresce green. Either PI or Hoechst 33342 dyes can be used as a nucleic acid counterstain and fluoresce red or blue respectively.[133,175] TUNEL has been used in a limited number of crustacean studies to assess DNA fragmentation, for example, DNA damage caused by viral diseases in black tiger prawn,[176–178] during the immune response of *Marsupenaeus japonicus*,[179] and during neurogenesis of *Homarus americanus*.[180]

In cryopreserved boar, human, mouse, and fish spermatozoa, DNA damage and alterations in downstream expression of specific genes involved in embryo development have been observed.[31,181–184] Sperm DNA is sensitive to external stressors such as radiation, toxins or temperature during spermiogenesis causing oxidative stress-induced genomic lesions.[132,185] Recently, sperm DNA integrity has been evaluated by examining genomic stability and expression of growth related genes during embryonic development.[132] Several modern technologies such as microarray analysis, quantitative real-time PCR analysis (qRT-PCR), next-generation sequencing (NGS) and bioinformatics have been used to assess variation in sperm genomic DNA and gene expression in aquatic vertebrates such as finfish.[31,132,186] Although the use of these more recent genetic technologies has not been documented in decapod crustaceans, the investigation of sperm DNA damage and downstream alteration of gene expression during early and late embryo development could be of value, particularly to burrowing decapods that may be exposed to heavy metals or toxins in sediment of aquaculture ponds or in their natural habitat.

Development of sperm quality assays aids the selection of high-quality male broodstock and allows for production of genetically superior offspring via both natural and artificial breeding programmes. Such diagnostic tools could also be used to assess post-thaw sperm quality during the development of cryopreservation and artificial fertilisation protocols for decapod crustaceans. A summary of traditional and advanced biomarkers of sperm quality and conditions that may improve male reproduction management is illustrated in Figure 1.

3 | SPERM CRYOPRESERVATION

Cryopreservation of crustacean sperm can facilitate the preservation and collection of high-quality male genetic material. This method permits frozen-thawed spermatozoa to be readily available to fertilise eggs whenever they are released, thus allowing powerful

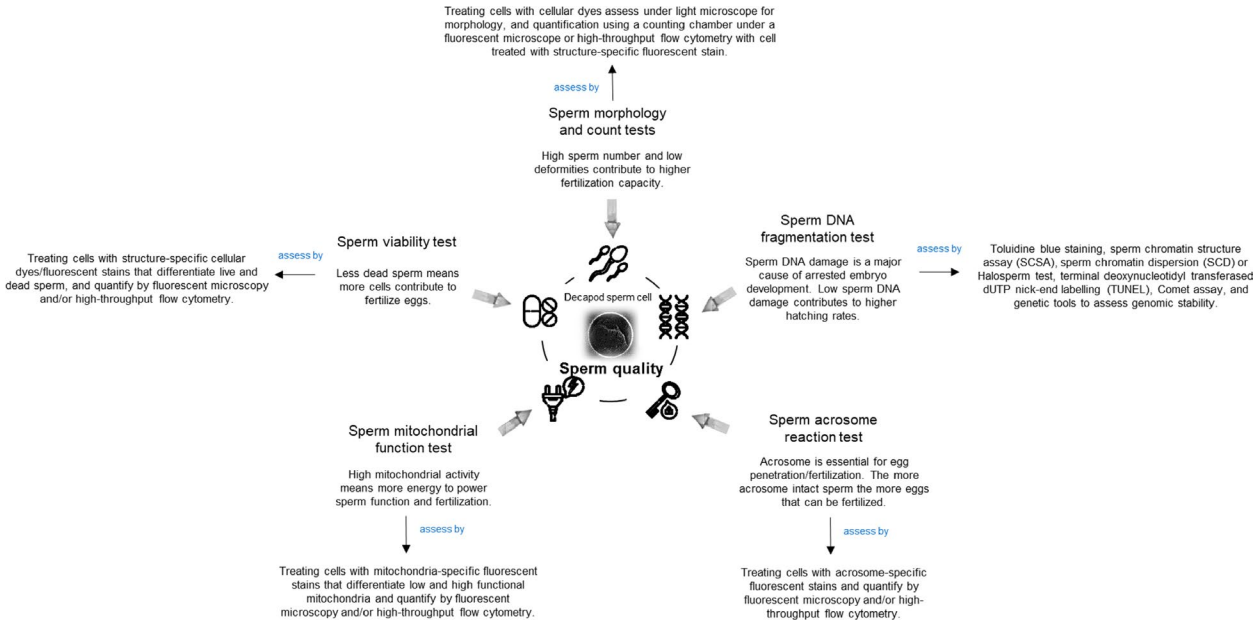

FIGURE 1 Traditional and advanced predictive biomarkers of sperm quality and conditions that may contribute to improved male reproductive performance

control of the timing of reproduction.[25] In addition, frozen spermatozoa can serve as a safe backup in situations where it is difficult to obtain fresh spermatophores or there is insufficient number of spermatozoa available for downstream applications like artificial fertilisation.[187] Finally, sperm freezing can preserve the germplasm of valuable genetic lines of founder/wild-caught individuals indefinitely, thereby permitting sustainable production of high-quality offspring over time.[26,50,187] Moreover, maintaining a sperm bank of high-quality males is more economic and efficient than maintaining significant numbers of male broodstock. Given these advantages, it is not surprising that sperm cryopreservation protocols have been established in some decapod crustaceans, primarily in marine shrimp (Table 2).

3.1 | Cryoprotectants

Cryopreservation employs low temperature to stop biochemical reactions and preserve living cells and tissues structurally intact. However, if not performed properly, freezing can damage cells via two distinct processes: ice crystal formation and concentrated solute effects.[188] Freezing injury is caused by ice crystals that mechanically pierce or tear apart the cells and intracellular structures.[189,190] Solute effects, which include chemical and osmotic gradients created by concentrated salts in the residual unfrozen liquid between ice crystals, cause cell injury through dehydration. Preferential freezing of water over solutes results in concentration of solutes/salts (a hypertonic state) inside the cell. This hyperosmotic stress can lead to shrinkage and eventually cell death.[190,191]

Cryoprotectants (antifreeze agents) are water soluble chemicals that decrease the melting point of water, and are commonly used to avoid cell injury caused by freezing.[189,190] Absence of cryoprotectants generally results in freezing of the entire water content inside the cell during cryopreservation, causing cell damage.[188,192] In order for a cryoprotectant to be effective and biologically compatible to living cells or tissues, it must possess following properties: (i) high soluble in water even at low temperature; (ii) capable of freely penetrating the cell membrane (except for non-penetrating cryoprotectants); and (iii) little or no toxicity at concentrations needed for cryopreservation.[192,193] Common cryoprotective agents include ethylene glycol, dimethyl sulfoxide (DMSO), glycerol and propylene glycol, and typically have a working concentration of 5%–15% in carrier solutions.[188,189,192-194]

Cryoprotectants can be cell permeating or non-permeating.[190,192] Permeating cryoprotectants are typically of small molecular weight (<100 Daltons) that facilitates easier penetration of cell membranes, making them osmotically inactive since they disperse equally in both extra- and intracellular spaces. This helps minimise the effect of excessive dehydration and ice crystal formation in cells during the freezing process.[190,192] Permeating cryoprotectants include DMSO, glycerol, ethylene glycol, methyl-formamide and dimethylformamide.[190,192] By contrast, non-permeating cryoprotectants, such as polyethylene glycol and polyvinylpyrrolidone, have larger molecular weights that prevent them from passing through the cell membrane.[190,192] These polymers are added to freezing solutions to also inhibit ice crystal formation. At the same concentration, non-permeating cryoprotectants are generally less toxic than the permeating ones.[188,192–194]

Use of cryoprotectants to freeze crustacean spermatozoa has been reported in several studies (Table 2). To date, ethylene glycol, dimethyl sulfoxide (DMSO), glycerol, glycine, $MgCl_2$, methanol, soy lecithin and trehalose have been the main cryoprotectants used

TABLE 2 Room temperature and chilled storage, long-term cryopreservation and vitrification of spermatophore and spermatozoa in decapod crustaceans

Species	Semen collection method	Preserved tissue/cells	Holding solution/cryoprotectant/cooling vessel	Equilibration time/dilution ratio
Freshwater crab				
Eriocheir sinensis	Manual extrusion	Spermatophore	Precooled (4°C) Ca^{2+}-free artificial seawater + 5% DMSO & 10% glycerol in cryovials	Not determined
Freshwater prawn				
Macrobrachium rosenbergii	Manual removal from female's sternum	Spermatophore	Artificial seawater + 42%–50% Ringer's solution in 50 ml vials	Not determined
Macrobrachium rosenbergii	Manual removal from female's sternum	Spermatophore	Artificial seawater + 42%–50% Ringer's solution in 50 ml vials	Room temp for 30 min; dilution ratio not mentioned
Macrobrachium rosenbergii	Electroejaculation	Spermatophore	20% Ethylene glycol added dropwise to a 2ml cryovial with a spermatophore.	Room temp for 15 min; dilution ratio not mentioned
Macrobrachium rosenbergii	Electroejaculation	Sperm	Sterile-filtered pond water + 10% DMSO + 10% propylene glycol	Room temp for 30 min; dilution ratio not mentioned
Macrobrachium acanthurus	Electroejaculation	Spermatophore	Distilled water + 10% & 20% glycerol or 10% methanol in 2 ml plastic microtubes	25°C for 10 min; dilution ration not mentioned
Marine crab				
Scylla serrata	Post-mortem dissection	Spermatophore, seminal plasma	Phosphate buffer (25 ml of 0.4 M NaCl/0.1 M glycine, 4 ml of 0.028 M NaH_2PO_4/0.072 M Na_2HPO_4 + cryoprotectants glycerol & DMSO + trehalose combination) in 0.5 ml semen straws	4°C for 16 h; 1 seminal plasma:4 diluent

Freezing rate & duration	Thawing conditions	Evaluation technique	Results	References
−1°C min^{-1} from room temp to −80°C; Plunged directly into −196°C	37°C for 3 min	Light microscopy	AR in spermatozoa is promoted by cryopreservation	[96]
Room temperature (20–25°C) for 17 h & 2°C for 4 days	Not mentioned	Artificial fertilisation (attached to female sternum using α-cyanoacrylate glue), fertilisation & hatching rates	Room temperature incubation resulted in 100% fertilisation & hatching success rates basing on the number of spermatophores fertilising eggs successfully ($n = 2$) Chilled incubation of spermatophores resulted to 72.7% fertilisation & hatching success rates basing on the number of spermatophores fertilising eggs successfully ($n = 11$)	[7]
10 min in LN_2 vapour then −196°C for 20 days	30°C; time not mentioned	Artificial fertilisation, fertilisation & hatching rates	100% females had fertilised eggs, all of which hatched	[6]
For −20°C cryopreservation: immediate storage at −20°C. For −196°C cryopreservation: equilibration at room temp for 15 min then cooled to −70°C at −1.5 to −2.5°C min^{-1} in a cooling container filled with 95% ethanol. Dry-ice cubes (1–2 cm^3) were slowly dropped in the 95% ethanol. At −70°C, vials were exposed to LN_2 vapour (−110 to −130°C) for 1–2 min before being plunged directly into LN_2 for 350 days	30°C for 5 min	Sperm viability via Trypan blue staining & fertilisation; light microscopy	10 or 20% glycerol or ethylene glycol at −20°C can store spermatophore for 10 days (80%–90% sperm viability). For longer cryopreservation (150 days), 20% ethylene glycol at −196°C is suitable (80%–90% sperm viability) with fertilisation rates of >60%	[203]
−1.5°C min^{-1} cooling rate between 27 & −39°C then stored in LN_2 for 90 days	35°C for 1 min	Eosin–nigrosin staining, light, electron & phase contrast microscopy	Cryopreserved spermatophores: 50.4 ± 1.9% sperm viability. Cryopreserved sperm: 28.3 ± 2.2% acrosome reactivity compared to 85.3 ± 2.5% in fresh spermatophores	[202]
−2°C min^{-1}; equilibration time not mentioned	30°C for 4 min	Eosin-nigrosin staining, light microscopy	Cold storage for up to 3 days: 35.3% sperm viability & 60%–73% sperm viability using the cryoprotectants Cooling rate at −2°C min^{-1} resulted to 21.8% sperm viability	[196]
−79 & −196°C for 30 days; straws were exposed to LN_2 vapour for 1 h then immersed & stored at −196°C in LN_2. For −79°C, straws were exposed to CO_2 for 1 h, & placed directly to dry ice	Room temp; thawing duration not mentioned	Eosin-nigrosin staining for sperm viability	Sperm viability at −79°C: glycerol = 93.2 ± 1.0% DMSO-trehalose = 93.0 ± 1.2% Sperm viability at −196°C: glycerol = 95.3 ± 1.4% DMSO-trehalose = 94.2 ± 0.0%	[205]

TABLE 2 (Continued)

Species	Semen collection method	Preserved tissue/cells	Holding solution/cryoprotectant/cooling vessel	Equilibration time/dilution ratio
Scylla serrata	Post-mortem dissection	Spermatophore, sperm	Ca^{2+}-free artificial seawater (FASW) + dropwise addition of glycerol (to 12.5%) in 0.5 ml straws sealed by polyvinyl alcohol	15°C for 10 min; dilution ratio not mentioned
Portunus trituberculatus	Post-mortem dissection	Sperm	Ca^{2+}-free saline + DMSO (percent not mentioned)	Not mentioned
Charybdis japonica	Post-mortem dissection	Sperm	Not mentioned + 15% DMSO in cryovials	Not mentioned
Charybdis japonica	Manual removal from female's spermatheca	Sperm	Sperm released from sperm mass using glass tissue grinder into Ca^{2+}-free saline + 15% DMSO in cryovials	Equilibration time not mentioned; 1 sperm mass:2 buffer
Scylla tranquebarica	Post-mortem dissection	Sperm	Ca^{2+}-free saline + 10% glycine in microcentrifuge tube	Room temp (25°C) for 60 min; 1 sperm suspension:3 cryoprotectant
Marine lobster				
Homarus americanus	Electroejaculation; Spermatophores cut in seawater & sperm mass extruded to form sperm suspension	Sperm suspension	Filtered seawater with 3–5 ml paraffin oil (Fisher, Saybolt viscosity 125/135) in Fisher culture tube	Not mentioned
Panulirus polyphagus	Post-mortem dissection	Sperm	Ca^{2+}-free saline + 10% glycine in 2 ml cryovials	Room temp (25°C) for 5 min; 1 spermatozoa:3 extenders
Marine shrimp				
Litopenaeus vannamei	Post-mortem dissection; Manual extrusion	Vas deferens	Filtered seawater or Ca^{2+}-free saline without cryoprotectant in 5 ml plastic vessel	15°C for 36 h
Fenneropenaeus indicus	Electroejaculation	Sperm	Filtered seawater (30 ppt) + Cryoprotectants: (a) 5% DMSO + 5% glycerol (b) 5% DMSO + 0.25 M trehalose in 5 ml cryovials	5 min at room temp, equal volume of cryoprotectant

Freezing rate & duration	Thawing conditions	Evaluation technique	Results	References
Programmed controlled-rate freezing at −5°C min^{-1} from different initial physiological temperatures (30, 23, & 15°C) to various subzero temperatures (−30 to −50°C) & subsequent storage at −196°C with cryoprotectants	55°C for 10–15 s	Artificial induction of acrosome reaction; hypo/hyperosmotic sensitivity tests for membrane integrity; trypan blue & eosin-nigrosin staining; light microscopy	DMSO, ethylene glycol & glycerol offered cryoprotection at 5%–12.5% v/v. Post-thaw sperm viability highest (52%) in 12.5% glycerol at a cooling rate of −5°C min^{-1}; 1% pronase releases sperm from spermatophores but can induce acrosome reaction thus mechanical shearing employed throughout experiments	[55]
Not mentioned	Not mentioned	Calcium ionophore A23187 treatment	Best preservation at 4°C	[262]
Not mentioned	Not mentioned	Light microscopy	83.8% sperm viability after 24 h preservation & 73.8% after 1 year preservation in LN_2. 25 min pre-freezing incubation was appropriate for sperm cryopreservation	[264]
Not mentioned	Not mentioned	Nα-benzol-DL-arginine-pnitroanilide (BAPNA) substrate method for acrosin activity, eosin B-staining, microscopy, SDS-PAGE	3 days cryopreservation at −196°C with 80.9 ± 1.0% sperm viability & 83.6 ± 1.7 μIU × 10^{-6} acrosin activity	[46]
Not mentioned	Not mentioned	Eosin-nigrosin staining, light microscopy	Cryoprotectant: 10% glycine + extender yielded 84.8 ± 1.0% sperm viability	[207]
4–7°C for 289 days	Not mentioned	Light & electron microscopy (EM)	Bacterial growth after >289 days at 4–7°C Sperm morphology using light microscopy. High-quality spermatozoa recovered after chilled storage <289 days	[199]
15 min at each of 25, 20, 16, 4, 2, −4, −20, −80, −150, and −196°C for 6 h	26°C for 30 s	Eosin-nigrosin staining, light microscopy	Highest sperm viability in 10% glycine at 91.9 ± 2.0% (5 min at room temperature equilibration time), 91.3 ± 2.6% (6 h at −20°C) & 75.9 ± 10.8% (6 h at −80°C). Best thawing at 26°C for 30 s with 76.1 ± 7.8% sperm viability	[206]
Not mentioned	Not mentioned	Phase-contrast microscopy	Seawater or Ca^{2+}-free saline useful for preparation & storage of gonadal tissue for gross morphology up to 36 h at 15°C Sperm viability after 36 h at 15°C was 65.2 ± 21.6% (seawater) & 51.0 ± 6.8% (Ca^{2+}-free saline) Sperm viability assessed morphologically; observing abnormal sperm	[271]
Room temp to −35°C at −1°C min^{-1}, then LN_2 vapour for 5 min before plunging into LN_2 for 60 days	20°C; thawing duration not mentioned	Egg water-induced acrosome reaction; sperm viability	70%–80% sperm viability after freezing at −35°C & −196°C	[273]

TABLE 2 (Continued)

Species	Semen collection method	Preserved tissue/cells	Holding solution/cryoprotectant/cooling vessel	Equilibration time/dilution ratio
Litopenaeus vannamei	Manual extrusion	Spermatophore, spermatic mass, sperm suspension	Sterile sea water (SSW; 35 ppt) + 10% methanol or glycerol or 5% ethylene glycol in 0.5 ml French straws sealed with Polyvinyl alcohol	60 min for spermatophore & spermatic mass; 15 min sperm suspension; 1 sperm mass:10 SSW
Penaeus monodon	Manual extrusion	Spermatophore	Mineral oil as extender with 0.1% penicillin-streptomycin without cryoprotectant in closed Eppendorf tubes (opened for 10 min every 7 days for oxygen transfer)	Not mentioned
Penaeus monodon	Manual extrusion	Spermatophore	Ca^{2+}-free 0.9% saline + 5% DMSO in cryovials	Spermatophore first in Ca^{2+}-free saline for 5 min, then transferred to cryoprotectant solution for 30 min
Litopenaeus vannamei	Manual extrusion	Spermatophore	Mineral oil with 0.1% penicillin-streptomycin without cryoprotectant in sterile Eppendorf tubes	Not mentioned
Penaeus monodon	Manual extrusion	Spermatophore	Ca^{2+}-free saline + 5% DMSO in cryovials	30 min at 25°C; dilution ratio not mentioned
Penaeus monodon	Manual extrusion	Spermatophore	Ca^{2+}-free saline + 5% DMSO in cryovials	30 min; dilution ratio not mentioned
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+}-free saline + 5% DMSO in cryovials	25°C for 30 min
Penaeus merguiensis	Manual extrusion	Spermatophore	Ca^{2+}-free saline + 15% $MgCl_2$ in cryovials	25°C for 15 min; dilution ratio not mentioned

Freezing rate & duration	Thawing conditions	Evaluation technique	Results	References
−0.5°C min^{-1} from room temperature to −32°C, then immersed in LN_2 for 3 days. Manual seeding done at −6°C	25°C for 40 s for spermatophores, 20°C for 10 s suspended in 0.2 M sucrose + SSW for sperm cells	Light microscopy; flow cytometry (DNA staining with propidium iodine)	Sperm viability after 2 h in vitro: SSW + 10% glycerol = 86.2 ± 4.5% SSW + 10% methanol = 85.6 ± 3.6% SSW + 5% ethylene glycol = 88.8 ± 1.1% Untreated SSW (control) = 87.0 ± 6.0% Sperm viability after 3 days: 61.6% using 10% methanol as cryoprotectant	[24]
2–4°C for 8 days	Not mentioned	Eosin–nigrosin staining; light microscopy	58.3 ± 2.9% viable sperm in mineral oil with 0.1% penicillin-streptomycin Fertilisation rate: 88.3 ± 0.9% Hatching rate: 87.6 ± 1.2%	[198]
Two-step freezing rate used: firstly −15°C min^{-1} from 25 to −10°C, then −2°C min^{-1} from −10 to −80°C using a controlled rate freezer. Thereafter cryovials plunged in LN_2 for 48 h	30°C for 2 min	Tissue weights; fertilisation; light microscopy	Frozen-thawed spermatophore has 79.7 ± 0.4% sperm viability with 79.9 ± 3.7% fertilisation rate & 87.8 ± 0.4% hatching rate	[8]
2–4°C for 35 days	Not mentioned	Eosin–nigrosin staining; light microscopy	Sperm viability (69.5 ± 3.9%) significantly higher ($p < 0.05$) among spermatophores preserved in mineral oil with 0.1% antibiotic compared with those preserved in mineral oil only (57.7 ± 3.4%)	[197]
One-step cooling rate at −2°C min^{-1} between 25 & −80°C then storage in LN_2 for 60 days	30°C for 2 min	Eosin-nigrosin staining; light microscopy; fertilisation rate	Spermatophores cryopreserved for <60–62 days: sperm viability: 87.3 ± 4.1%, fertilisation rate: 71.6%–72.2%, hatching rate: 63.6%–64.1% Long-term storage of spermatophores: sperm viability: 53.3 ± 4.3% (after 90 days), 46.7 ± 4.2% (120 days), <40% (210 days) One-step cooling rate at −2°C min^{-1} resulted in 93.3 ± 2.7% sperm viability	[9]
One-step cooling rate at −2°C min^{-1} between 25 & −80°C then storage in LN_2 for 210 days	37°C for 2 min with cryovials wiped with 70% EtOH	Bacterial assays over time	Cryopreservation of spermatophores eliminated pathogenic bacteria during long-term storage in LN_2	[204]
−2°C min^{-1} from room temperature to −80°C, maintained for 2 min, then plunged in LN_2 for 70 days	30°C for 2 min	Flow cytometry; eosin-nigrosin staining; light microscopy	Highest sperm viability of 34.4 ± 3.4% using this protocol followed by 33.3 ± 3.9% at −1°C min^{-1} freezing rate Long-term cryopreservation: 30 min equilibration at 25°C in 5% DMSO yielded 49.5 ± 8.3% sperm viability in equilibrated sample, 44.3 ± 6.6% viability in samples frozen 1 day, & 33.0%–37.0% viability after 10, 20, 30, 40, 50, 60 & 70 days	[54]
10 min at each of 25, 20, 16, 4, 2, −4, −20, −80, −150°C for 90 days	27°C for 2 min	Eosin-nigrosin staining; light microscopy; fertilisation rate	Spermatophore cryopreserved with good sperm viability (55.4 ± 0.3%), fertilisation (64.1 ± 2.1%) & hatching (62.5 ± 1.5%) rates Equilibrium time in $MgCl_2$ yielded 88.2 ± 7.3% viable sperm	[201]

TABLE 2 (Continued)

Species	Semen collection method	Preserved tissue/cells	Holding solution/cryoprotectant/cooling vessel	Equilibration time/dilution ratio
Litopenaeus vannamei	Manual extrusion	Sperm	Hank's balanced salt solution + antibiotic/antimycotic without cryoprotectants in microtubes	23°C for 12 h; 1:4 dilution
Litopenaeus schmitti	Post-mortem dissection	Sperm mass	Ca^{2+}-free saline + 5 % glycerol in cryovials	10 min; dilution ratio not mentioned
Litopenaeus vannamei	Manual extrusion	Sperm	Sterile seawater; Activation: Freezing solution (5% egg yolk & 0.2 M sucrose) + 5% DMSO or ethylene glycol in cryovials	30 min; dilution ratio not mentioned
Litopenaeus vannamei	Manual extrusion	Sperm	Ca^{2+}-free saline + 30% methanol + 0.4 M trehalose + 2% soy lecithin in microtubes	25°C for 10 min; dilution ratio not mentioned
Litopenaeus vannamei	Manual extrusion	Spermatophore	Ca^{2+}-free saline + 0.4 M trehalose + 2% soy lecithin in microtubes	25°C for 10 min; dilution ratio not mentioned
Penaeus monodon	Electroejaculation	Sperm	Ca^{2+}-free saline + 5% DMSO in cryovials	Room temp for 30 min; dilution ratio not mentioned
Fenneropenaeus indicus	Manual extrusion	Sperm	Ca^{2+}-free saline + 5% DMSO + 5% MeOH + 10% egg yolk as co-cryoprotectant & 0.25 M trehalose in cryovials	Room temp for 30 min; dilution ratio not mentioned

either alone or in combination with concentrations ranging from 5 to 20%.[34,190,195] These cryoprotectants were mixed with carrier solutions such as filtered fresh or seawater, Ringer's solution, or phosphate buffered and Ca^{2+} - free saline. Spermatophores or spermatozoa usually were equilibrated with cryoprotectants for 5–60 min, at a ratio of 1:2, 1:3 or 1:4 spermatic mass to freezing solution, depending on species (see Table 2).

3.2 | Room temperature and chilled storage of crustacean spermatozoa

The spermatophore, spermatic mass or spermatozoa of crustaceans have been held at room temperature (20–25°C), chilled (2–4°C) or cryopreserved in liquid nitrogen (−196°C).[7,10,196] For short-term storage of decapod spermatozoa, most studies reported good sperm viability in the absence of cryoprotectant. Short-term storage (4–17 h) of spermatophores at room temperature in Ringer's or Ca^{2+}- free saline without cryoprotectant maintained greater than 50% sperm viability, fertilisation and hatching rates in both freshwater prawn (*Macrobrachium rosenbergii*)[7] or Pacific white shrimp (*L. vannamei*) respectively.[10] Spermatophores from black tiger prawn and Pacific white prawn kept at 2–4°C in mineral oil with 0.1% penicillin-streptomycin but no cryoprotectant exhibited 60% sperm viability and 80% fertilisation and hatching rates after for 35–42 days.[197,198] Moreover, clawed lobster (*Homarus americanus*) spermatophores preserved for up to 289 days in paraffin oil without cryoprotectant at 4–7°C maintained normal morphology as determined by both phase-contrast and electron microscopy.[199] However, storage for 3 days or longer at 2°C in distilled water containing 10%

Freezing rate & duration	Thawing conditions	Evaluation technique	Results	References
23°C for 4 h	Not mentioned	Fertilisation rate	92.0% viable sperm using modified artificial extenders with 60.4% female successfully spawned	[10]
Two-step freezing protocol: 25 to −6°C at −2°C min^{-1}, then −6 to −32°C at −0.5°C min^{-1}, then sperm mass immersed in LN_2 for 15 days	20°C for 10 s	Eosin-nigrosin staining & light microscopy	5% glycerol yielded 17.2 ± 0.8% viable sperm after 30 days. Two-step freezing protocol achieved 42.9 ± 0.6% viable sperm after 15 days in LN_2	[285]
Not mentioned	25°C for 40 s	Eosin-nigrosin staining & light microscopy, flow cytometry	Short-term storage yielded 20%–40% sperm viability after 30 days in LN_2	[94]
Room temperature (25°C) for 10 min then vitrified directly in LN_2	1 ml Ca^{2+}-free saline; duration not mentioned	Eosin–nigrosin staining, fluorescent microscopy	30% methanol for 120 min yielded 79.5 ± 1.3% sperm viability. Trehalose ± soy lecithin is an effective extracellular cryoprotectant for vitrification yielding 88.0 ± 1.6% sperm viability after 120 days	[195]
Vitrified directly in LN_2 for 150 days	36°C for 1 min	Light & fluorescent microscopy	91.8 ± 3.0% sperm viability using cryoprotectant with 73.0 ± 2.6% fertilisation rate but 0.0% hatching rate in all treatments	[34]
25°C to −10°C at −15°C min^{-1}, then −10 to −80°C at −2°C min^{-1}, after which cryovials plunged into LN_2 for 180 days	28°C for 2 min	Comet assay, sperm chromatin dispersion test (SCDt)	(1) % SDF was strongly correlated between SCDt & two-tailed comet assays (Pearson $r = 0.989$; $p = 0.01$) (2) % SDF did not increase due to mechanical stress induced by vortexing ($p = 0.76$) (3) % SDF was higher in domesticated (6.8 ± 4.5%) than wild (3.3 ± 1.5%) male broodstock ($p < 0.001$)	[157]
−0.5°C min^{-1} between 4°C & −80°C, hold for 5 min before storage in LN_2 for 45 days	30°C for 1 min	Eosin-nigrosin staining; light microscopy; HOST & DNA integrity analyses; fluorescent microscopy	Sperm viability was 83.8 ± 2.5% after one-step slow freezing of spermatophores in 5% DMSO + 5% MeOH. Sperm quality was great at a freezing rate of −0.5°C min^{-1} with 53.9 ± 4.9% sperm viability, 45.6 ± 4.2% HOST & 58.1 ± 1.7% DNA integrity	[81]

glycerol led to low viability (35%) in giant freshwater prawn spermatozoa, which was attributed to the toxicity of the cryoprotectant.[196] These reports suggest that the use of cryoprotectants may be inappropriate for short-term storage of crustacean sperm above 0°C, due their toxic effect on metabolically active cells.[200]

3.3 | Long-term cryopreservation

Using liquid nitrogen, spermatophores and spermatozoa of crustaceans have been reported to be preserved for between 90 and 180 days depending on the species and cryoprotectant employed.[157,201–203] Unlike vertebrate spermatozoa, sperm viability across several species of crustaceans appears to decline significantly beyond 180 days of storage.[9,190] For crustaceans, the rate of cooling spermatozoa prior to freezing varies depending on species and generally ranges from −5 to −2.5°C min^{-1}, typically resulting in greater than 50% viable spermatozoa (see Table 2). In most cases, frozen spermatophores or spermatozoa can be thawed at 20–30°C for 30 s to 5 min depending on the species (see Table 2). In giant freshwater prawn, the best cooling rates for spermatozoa reportedly range from −1.5 to −2.5°C min^{-1} using either 10% DMSO, 10% propylene glycol,[202] 10% glycerol or 20% ethylene glycol as cryoprotectants.[203] Their spermatozoa were subsequently best thawed in a 30–35°C water bath for 1–5 min.[6,202,203] These procedures resulted in the cryopreservation of spermatozoa at −196°C for 90–150 days with more than 50% sperm viability.[202,203] The most frequently used cryoprotectant for penaeid shrimp sperm cryopreservation is 5% DMSO using either one- or two-step freezing rates. One-step freezing normally uses a rate of −2°C min^{-1} between 25 and −80°C before

storing in liquid nitrogen (LN_2).[9,204] Two-step freezing involves lowering of temperature at −15°C min^{-1} from 25 to −10°C, then at −2°C min^{-1} from −10 to −80°C. Thereafter, cryovials are plunged in LN_2 at −196°C.[8,157] Frozen spermatozoa are thawed in a 27–30°C water bath for 2 min.[8,9,157,201,204] Using these procedures, more than 50% sperm viability can be achieved following 90–180 days of cryopreservation.[8,9,157,201,204] In giant mud crab (*Scylla serrata*), spermatozoa can be cryopreserved in LN_2 using 15% glycerol or 5% DMSO and 0.25 M trehalose as cryoprotectants for up to 30 days with more than 90% sperm viability.[205] In another study, giant mud crab spermatozoa exhibited 50% viability after 8 h frozen in LN_2 at a cooling rate of −5°C min^{-1} using 12.5% glycerol as the cryoprotectant, and thawing in a 55°C water bath for 10–15 s.[55] In mud spiny lobster (*Panulirus polyphagus*), spermatozoa exhibited up to 80% viability after 24 h cryopreservation using 10% glycine as cryoprotectant and a freezing rate of 15 min at each of 8 graded temperatures (25, 20, 16, 4, 2, −4, −20, −80 and −150°C), followed by immediate storage in LN_2, and thawing at 26°C for 30 s.[206]

Post-thaw sperm quality is usually evaluated by measuring their fertilisation and hatching rates in crustaceans.[6,8,9,203] In giant freshwater prawn, spermatophores equilibrated in freshwater for 15–30 min after thawing were attached to the female's sternum using α-cyanoacrylate as a glue, resulting in successful fertilisation and hatching (though the authors did not report the rates.[6] In black tiger prawn, two studies reported 70%–89% fertilisation rates and 63%–88% hatching rates after artificial fertilisation using cryopreserved spermatophores.[8,9] In summary, both short- and long-term storage of sperm at room temperature, by chilling or cryopreservation in liquid nitrogen has been reported and appear relatively successful in different taxonomic groups of crustaceans, including freshwater prawn and marine shrimp, crabs, and marine lobsters.[6–9,157,197–199,201–204,206,207] Nevertheless, further work in this area to optimise freezing protocols for other decapod crustaceans is required.

3.4 | Sperm vitrification

An alternative to conventional sperm cryopreservation is the rapid freezing of spermatozoa by a process known as vitrification. This process, through an extreme increase in viscosity during freezing, causes solidification of liquid into an amorphous or glassy state at low temperature to avoid inducing ice formation and crystallisation.[189,192,208] If freezing of viscous solution occurs rapidly, the supercooled liquid retains its physical liquid properties until it reaches its glass-state transition temperature. Rapid freezing below this temperature, maintains the disorderly organisation of the solution's molecules but its physical properties remain rigidly solid. In this state, molecules are locked in place as if the liquid were frozen in time, resulting in a 'solid liquid' known as 'glass'.[209]

For the vitrification process, cells need to be exposed to a high concentration of cryoprotectant solution early. Rapid cooling allows the entire volume of a cell to change to a glassy solid state (vitrifying), devoid of freezing.[189,192,193,208,210] In decapods, whiteleg shrimp spermatozoa were equilibrated for 10 min at room temperature (25°C) in several different cryoprotectant solutions: (i) Ca^{2+}- free saline +0.4 M trehalose (base solution), (ii) base solution +30% methanol (MeOH), (iii) base solution +30% MeOH +1% soy lecithin, (iv) base solution +30% MeOH +2% soy lecithin, (v) base solution +1% soy lecithin and (vi) base solution +2% soy lecithin, before plunging them directly into LN_2 (−196°C).[195] Results showed that trehalose alone (base solution; 90.1 ± 2.4% sperm viability) or trehalose +2% lecithin (91.1 ± 3.9% sperm viability) were effective extracellular cryoprotectants for vitrification after 120 days storage in LN_2.[195] In a subsequent study, whiteleg shrimp spermatozoa exceeded 90% viability after 150 days in LN_2 using 0.4 M trehalose +2% lecithin as the cryoprotectants.[34] However, artificial fertilisation with thawed spermatozoa preserved by this method resulted in extremely poor to zero hatching rates, suggesting that the vitrification process may have induced high rates of sperm DNA damage (but this remains to be determined -emphasising the need to develop sperm DNA damage assays for crustaceans). Clearly, application of conventional slow-freezing or vitrification techniques to the aquaculture industry would revolutionise the maintenance of male broodstock genetics in sperm banks and by so doing, significantly reduce operational expenses associated with broodstock management. Coupled with the development of successful techniques to efficiently reinfuse these genetics back into broodstock females via procedures such as artificial fertilisation, such advanced reproductive technologies could provide better control of reproduction and selective breeding of valuable genetic lines of economically important decapod crustaceans.

4 | NATURAL AND ARTIFICIAL FERTILISATION IN DECAPOD CRUSTACEANS

Understanding the physiological changes to gametes during the process of fertilisation in decapod crustaceans is essential for improving hatchery management, selective breeding and other reproductive strategies. Changes in sperm physiology and mating behaviour are particularly well documented in freshwater crayfish,[3,98,211] therefore, the subsequent description will primarily focus on this group of decapod crustaceans. During mating, the thick muscle of the distal vas deferens contracts and a segment of the sperm cord (a continuous cord containing spermatozoa surrounded by primary and secondary secretions) is squeezed through the gonophores and ejected by the *appendices masculinae*. The internal sperm cord is fragmented upon extrusion releasing an individual spermatophore unit. Only one spermatophore is transferred to a female's sternum at a time and forms a sticky opalescent mass within 10 min post-extrusion. By 1 h post-extrusion, the spermatophore begins to harden, and completely solidifies within 24 h. At this point, the female crayfish has already separated from the male and extends her pleon, folds her abdomen and expands her uropods to create a temporary brood chamber. Eggs

are visible within the chamber and some are already attached to the pleopod setae, with all eggs firmly attached after 48 h. Hydration of the attached spermatophore occurs 24–48 h post-mating; confirmed by increased swelling and coiling of the sperm cord thereby increasing its size.[64] After 72 h, the spermatophore softens and between 72 and 96 h post-mating, many sections of the sperm cord begin to coalesce. Fissures are observed in the matrix of the secondary spermatophore layer from 48 h, which increase further after 72–120 h post-mating. Ultimately, the spermatophore disintegrates completely between 96 and 120 h.[64] After mating, the female releases a secretion from her glair glands that dissolves the wall of the spermatophore, allowing her to smear spermatozoa across her ventral abdomen before egg release. Glair secretions are sufficient to firmly hold spermatozoa and prevent them being washed away, until fertilisation is complete.[80] The female regulates the timing of sperm release by breaking open the primary spermatophore layer and drawing spermatozoa into her brood chamber to facilitate fertilisation.[64,212] The spermatophore is completely ruptured after egg release and attachment to the pleopods, enabling the female to manipulate spermatozoa into her brood chamber to complete external fertilisation.[64] The female brood chamber is maintained to incubate fertilised eggs for up to 5–7 days post-oviposition. After this time, her abdomen is extended to gently ventilate the visibly fertilised eggs using her swimming legs.[64] Time of natural mating and egg-laying differs across decapod crustaceans depending on water temperature but this can be controlled artificially by manipulating temperature and photoperiod.[212–214]

4.1 | Spermatophore structure and function

Across decapod crustaceans, the spermatophore varies morphologically and can be classified into three general types. The first type forms a small round or ellipsoid shape usually found in brachyuran crabs (eg blue-swimmer crabs and giant mud crabs). This type of spermatophore is suspended in seminal fluid and deposited in the female spermatheca during true copulation. In brachyuran crabs, spermatophores degenerate after copulation and lose their protective function, only serving to keep spermatozoa together during transfer to the female prior to internal fertilisation.[39] The second type is the pedunculated spermatophore usually found in anomuran crabs (eg hermit crabs, mole crabs and sand crabs). This type of spermatophore forms a stalk or peduncle that is fixed onto the ventral sternum of the female during copulation, where it remains for an extended period until the female mechanically manipulates it for external fertilisation.[39] The third type of spermatophore, produced by most macrurans (eg crayfish and lobsters), contains spermatozoa enclosed within a sperm cord surrounded by one or more layers of protective gelatinous matrix. This type of spermatophore is also produced by penaeid shrimps but with accessory structures such as attachment wings.[39] Generally, this type of spermatophore is deposited on the ventral sternum of female crayfish and lobsters or inserted in the thelycum (sex organ) of female penaeid shrimp as well as homarid and nephrosis lobsters.[39] In freshwater crayfish, spermatophores harden upon exposure to water. This hardening protects spermatozoa from physical damage brought by environmental stressors, thereby enhancing sperm viability.[64,80,98,215] Hardening of the secondary spermatophore layer is caused by enzymatic reactions associated with calcification of chitin complexes. Moreover, highly acidic mucopolysaccharides (containing chondroitin sulphate and hyaluronic acid) contribute to the calcification and antimicrobial activity of the spermatophore. Chondroitin sulphate aids desiccation of spermatophores by providing elasticity and resistance to compression.[39,80,216] Calcium-related proteins such as sarcoplasmic calcium-binding protein, crustacean calcium-binding protein 23, ryanodine receptor, and troponin C2 have been identified in the spermatophore of freshwater crayfish and are suspected to be responsible for calcification during hardening.[80,98,217–219]

4.2 | Natural fertilisation

Spermatozoa encased by a spermatophore are unable to fertilise, only after chemical 'activation' do they acquire the capacity to fertilise; a process that is time-dependent.[220,221] These morphological and molecular changes are known as sperm capacitation.[80,98,219,222] Sperm capacitation in crustaceans occurs either inside seminal receptacles such as the thelycum in Penaeid shrimp, or on the ventral portion of the female, such as in freshwater crayfish.[39,98,223,224] During the process, both spermatophores and spermatozoa alter their morphology. In freshwater crayfish for example, the release of glair gland secretions by the female to dissolves the hard wall of the spermatophore is known to initiate sperm capacitation prior to fertilisation.[80] Changes to the spermatozoon occur primarily in the extracellular capsule, plasma membrane and subacrosomal zone after the spermatophore is dissolved. During capacitation, the extracellular capsule swells and space appears between the spermatozoa and the capsule. The anterior portion of the acrosome changes from a single- to a multi-layered structure and the plasma membrane wrinkles, thus increasing the wrapping of membrane around the acrosomal apical cap.[80,98] After capacitation, the acrosome reaction involves morphological and molecular changes to the acrosomal vesicle and discharge of subacrosomal and nuclear material into the egg.[80,97,98] The subacrosomal zone detaches from the main acrosome, losing electron density, and membranous lamellae separate from the free spermatozoon. Electron-dense material, that wrap the filaments in the innermost portion of the acrosome pre- and post-mating, are released from the spermatozoon (Figure 2).[80] The discharge of subacrosomal contents is aided by its morphological alteration during the process. The subacrosomal zone is calcified, thus increasing its ability to puncture the egg membranes in order to successfully transfer nuclear material into the egg.[98,217] Discharge of the inner nuclear material occurs simultaneously with the acrosome filaments, forming a droplet or filament structure in the anterior portion of the spermatozoon.[80,98,218] After discharge of the nuclear material, the extracellular capsule, membranous lamellae and plasma membrane are eliminated. Nuclear

material at this time is less condensed than in spermatozoa at earlier stages.[98,217] Transfer of nuclear material inside the egg activates an instantaneous electrical block to protect the egg from polyspermy - fertilisation by multiple spermatozoa.[225–228]

In other decapod crustaceans such as black tiger prawn (*P. monodon*) and pink prawn (*F. paulensis*) that have a closed thelycum, capacitated spermatozoa have a more electron-dense acrosome and subacrosomal zone and less condensed nucleus.[229,230] Similar to

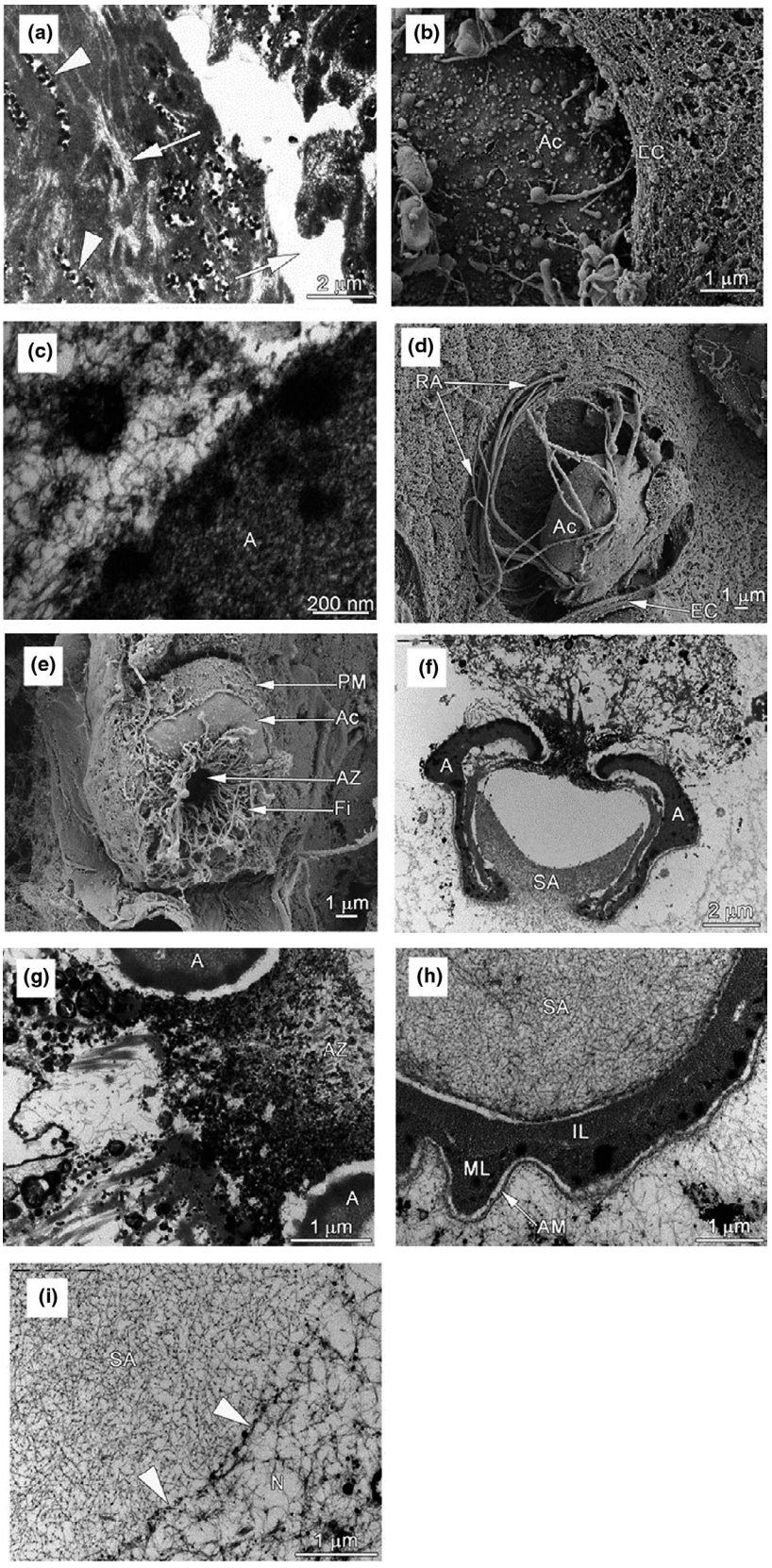

FIGURE 2 Electron (transmission and scanning) micrographs of the spermatophore wall and released spermatozoa in narrow-clawed crayfish (*A. leptodactylus*). (a) dissolving wall of the spermatophore with distorted granules (arrowheads) and areas where female glair secretions have penetrated (arrows); (b) exposed anterior acrosome secreting its contents in numerous droplets; (c) sagittal close-up showing acrosomal secretion with electron-dense vesicles at the inner surface of the acrosome being discharged extracellularly (d) a spermatozoon released from the extracellular capsule with nuclear radial arms and acrosomal complex clearly visible; (e) discharge of filaments from the anterior portion of the acrosome causing a cavity; (f) sagittal section of a spermatozoon extruding filaments and electron-dense material from the apical zone of the acrosome leaving a withdrawn subacrosomal zone; (g) close-up of electron-dense material being expelled via the apical zone; (h) cross-section of remaining acrosome layers and subacrosome zone after release of electron-dense materials; (i) boundary between subacrosome zone and nucleus (arrowheads). A, acrosome; Ac, acrosome complex; AM, acrosomal membrane; AZ, apical zone; EC, extracellular capsule; Fi, filaments; IL, inner acrosome layer; ML, middle acrosome layer; N, nucleus; PM, residual plasma membrane; RA, radial arms; SA, subacrosomal zone[80]

sperm capacitation in mammals, changes in protein composition of the plasma membrane and increasing protein tyrosine phosphorylation have been observed.[230,231] After the acrosome reaction, the apical cap becomes less concave.[229,230] In decapods with open thelycum such as the whiteleg shrimp (*L. vannamei*), copulation is accomplished by attaching the male spermatophore onto the surface of the thelycum 4–6 h before spawning. After attachment, ultrastructural changes associated with sperm capacitation involve the formation of a filamentous meshwork between sperm nucleus and hemispherical cap.[223,232] Most reports describing the acrosome reaction in shrimp spermatozoa are based on artificial induction by egg water *in vitro* and, as such, are not as specific as *in vivo* acrosome reaction reported in freshwater crayfish.[107,223,232] In black tiger shrimp, a mature spermatozoon consists of three major regions: anterior, middle, and posterior. An acrosomal spike is visible on the anterior region while the posterior region contains the nucleus and forms the main body of the spermatozoon. The subacrosomal materials occur in the middle region. During egg water-induced acrosome reaction *in vitro*, the acrosome loses its spike (termed depolymerisation) followed by acrosomal exocytosis of nuclear materials.[49,105,233] Upon sperm contact with the vitelline egg envelope during natural fertilisation, the acrosomal spike also degenerates, and an electron-dense spherical mass is formed.[49,105] The spherical mass draws the nuclear material into the egg's cytoplasm during sperm entry.[49]

In crabs, acrosome reaction occurs simultaneously with sperm penetration into the oocyte membrane.[233] Once the acrosome reaction is complete, a fertilisation cone is formed at the site of sperm-egg contact. Between the acrosome tubule and the fertilisation cone, bell-shaped corpuscles are observed that are believed to release a binding-like substance to stimulate sperm-egg membrane fusion.[233,234] Since sperm morphology varies across different species of decapod crustaceans, our general understanding of the process of natural fertilisation is still largely inadequate. In particular, the molecular mechanisms underlying sperm capacitation, acrosome reaction and fertilisation of eggs requires further research, that will ultimately improve our ability to develop successful artificial fertilisation techniques.

4.3 | Artificial fertilisation

In traditional aquaculture of economically important crustaceans, animals might only reach sexual maturity and reproduce in captivity when conditions are favourable such as the black tiger prawn. Moreover, males and females of some aquaculture species may not mature and reproduce simultaneously in captivity.[235] With intensification of aquaculture, interest in controlling crustacean reproduction using assisted breeding techniques has gained great impetus.[25,28] With such techniques, gametes can be collected from superior broodstock with high-quality phenotypic traits and used for artificial fertilisation during a precisely controlled time window,[235] ensuring genetic selection for high-quality offspring can be maximised for commercial production.

Artificial fertilisation (AF) in crustaceans involves either the introduction of sperm extruded from the male into the female sex organ for internal fertilisers, or adhesion of the spermatophore near the ventral gonophores of the female for external fertilisers.[235] AF is a means of reproductive control that allows fertilisation to occur anytime, which should dramatically speed up selective breeding programmes while dramatically reducing the cost.[235] Due to unique differences in reproductive physiology, fertilisation and hatching rates after AF varies in each species of decapod.[28,233] Although previous studies reported high spawning rates, they yielded few or no embryos.[6,18] Despite not yet being widely employed, the external nature of fertilisation in many decapods, makes them ideal candidates for AF, which has been attempted and reported in a number of species. For example, for freshwater prawn AF, spermatophores were extruded by post-mortem dissection,[236] manual extrusion,[5] electroejaculation,[4,44] or direct removal from the female's sternum or seminal receptacles post-copulation.[6,7] Generally, both fresh and cryopreserved spermatophore can be utilised for artificial fertilisation.[28,203] Female freshwater prawns were fertilised artificially using a retainer tube with their ventral side facing the operator.[6,7,236] AF efficiency was evaluated through sperm counts, fertilisation and hatching rates of eggs, and was highly successful in laboratory experiments in freshwater prawn and penaeid shrimp.[9,28,203] Using AF, 75%–100% female freshwater prawns had fertilised eggs with 90%–100% hatching rates using either fresh or cryopreserved spermatophores.[6,7] In one study, fertilising capacity was determined by evaluating the survival rate of developing embryos 5 days after spawning, as well as estimating the rate of effective spermatophores (spermatophores that yield normal hatching of fertilised embryos) after adhesion.[203] High embryo survival rates (75%–90%) were observed using cryopreserved spermatophores stored in LN_2 for 30–100 days.[203] Moreover, high male fecundity

after AF appeared directly proportional to sperm concentration in a given spermatophore, demonstrating the direct link between sperm quality and egg fertilisation and hatching rates. By contrast, in narrow-clawed freshwater crayfish, freshly electroejaculated spermatophore with 80% sperm viability failed to fertilise eggs nor produce offspring, suggesting that electroejaculated spermatophores in this study may have contained significantly fewer spermatozoa to participate in fertilisation.[18] Furthermore, white-leg shrimp spermatozoa with >90% viability after spermatophore vitrification achieved >70% fertilisation rates but had low to no hatching after artificial fertilisation.[34] The inability of these embryos to survive until hatching was thought to be caused by fertilisation with vitrification-induced DNA-damaged spermatozoa, but this claim remains to be validated.[34] These above preliminary studies offer hope for the commercial development of AF in selected decapods, while for others greater understanding of the underlying mechanism and timing of gamete interaction during fertilisation is required before sperm cryopreservation and AF can be of benefit to their commercial aquaculture.

5 | CONCLUSIONS

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

Application of conventional sperm cryopreservation or vitrification techniques to crustacean aquaculture would revolutionise the maintenance of male broodstock genetics in Sperm Banks, and by so doing, significantly reduce operational

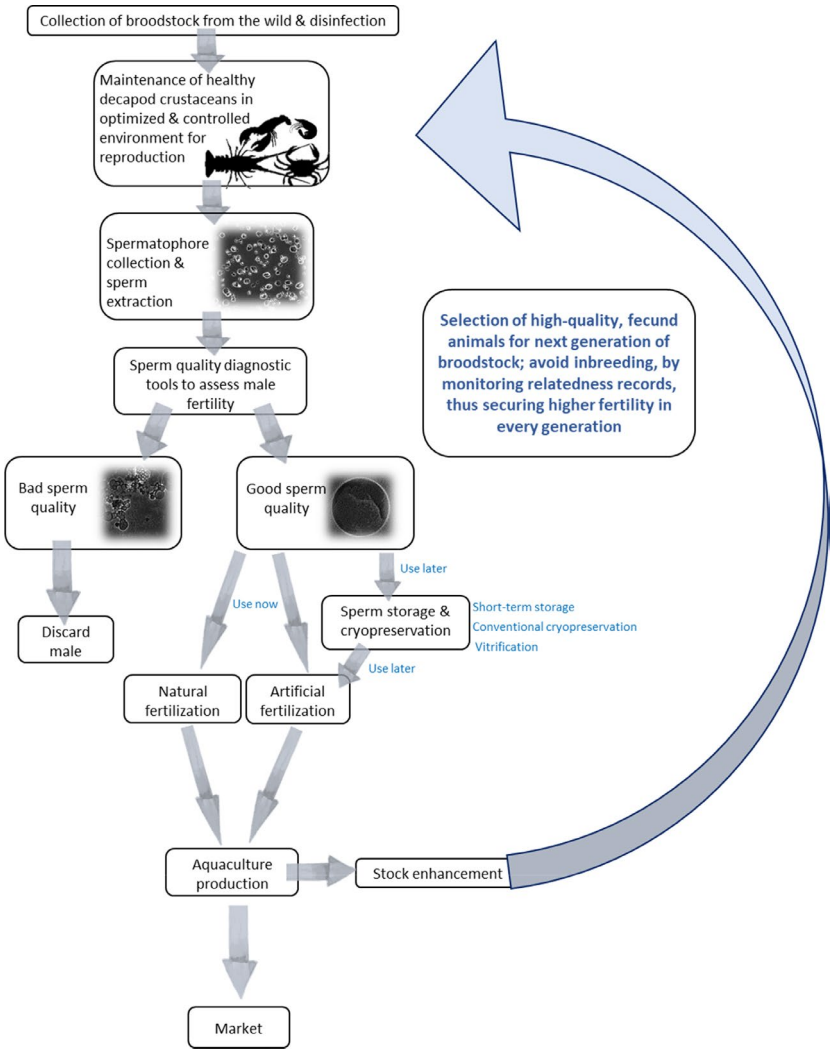

FIGURE 3 Application of advanced reproductive tools to improve sustainable production of economically important decapod crustaceans in aquaculture

expenses associated with broodstock management. Coupled with techniques to reinfuse these genetics back into broodstock females via procedures such as artificial fertilisation, these advanced reproductive technologies will provide better control of reproduction and selective breeding of valuable genetic lines of decapod crustaceans.

Advanced reproductive tools to assess male fertility can be further used as sensitive biomarkers to improve sperm handling procedures, broodstock husbandry and nutrition. Successful application of these advanced diagnostic tools to decapods could help identify the impact male broodstock with low sperm quality may have on poor offspring yield or survival, particularly in the case of low sperm quality caused by factors such as DNA damage that is undetectable by traditional methods, thereby allowing breeders to make informed decisions about which males to discard in their broodstock. Males identified with high-quality spermatozoa can be retained for natural breeding or their spermatozoa used for downstream cryopreservation and artificial fertilisation. In turn, improving overall reproductive performance, productivity and cost-efficient management in the hatchery. In addition, formulation of broodstock diet and adjustment of environmental conditions to optimise male reproductive performance can be achieved through evaluation of sperm quality with the application of advanced diagnostic tools specific for decapods. Production of decapod crustaceans can be substantially improved by careful selection of broodstock males at each successive generation based on both physical (eg absence of body damage, appropriate size at sexual maturity, body pigmentation, complete appendages, disease resistance, growth and moulting rates) and reproductive (sperm quality) traits (Figure 3). These technologies can help breeders make better decisions about broodstock management leading to greater numbers of superior progeny, thus accelerating both commercial crustacean aquaculture production and stock enhancement programmes.

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No data were generated for this review.

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