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Quantitative determination of ovarian development in penaeid prawns (Decapoda: Penaeidae)

Jason A. Crisp[1], Frances M. L. D'Souza[2], James R. Tweedley[1], Gavin J. Partridge[1,3], Navid R. Moheimani[1]

[1]School of Veterinary & Life Sciences, Murdoch University, Perth, Australia;

[2]Department of Water, Perth, WA, Australia 6000; and

[3]Australian Centre for Applied Aquaculture Research, Fremantle, WA, Australia 6160

Abstract

We developed histological methods to increase precision in measuring oocyte size and, for the first time, quantify changes in oocyte composition during ovarian development in penaeids. Wild-caught female *Metapenaeus dalli*Racek, 1957 from the Swan-Canning Estuary, Perth, Western Australia were used as a model species to compare the novel method to traditional techniques. Morphological analysis showed that ovarian development in *M. dalli* occurs in five stages: immature, early maturing, late maturing, mature, and post spawning, which is consistent with other penaeids. Analysis of key morphometric parameters of length and Gonad Somatic Index (GSI) showed that GSI provided the strongest discriminators of ovarian development. Oogenesis was similar to qualitative descriptions of other penaeid prawns and most-closely related to previous descriptions of *Metapenaeus affinis* Milne Edwards, 1837. Comparisons between the novel perimeter tracing and traditional single linear methods for measuring oocyte dimensions showed that greater precision was achieved by tracing. This resulted in a 17–40% reduction in the confidence limits of the means for all cell types measured. A novel histological technique of examining oocyte composition was also developed. This technique allowed for the relationship between stages of ovarian development and proportion by volume of

oocyte types to be determined. The difference in the proportions of cell types between each stage of ovarian development was found to be statistically significant, except between immature and post spawning females. The novel methods developed in this study provide new opportunities in the study of ovarian development in penaeids and possibly in other species.

Keywords: aquaculture, histology, Metapenaeus dalli, oogenesis, reproduction

Introduction

The western school prawn *Metapenaeus dalli*Racek, 1957 is a penaeid that occurs in inshore marine waters < 30 m in depth, from southern Java (Indonesia) and along the western coast of Australia from Darwin in the north to Cape Naturaliste in the south (Gray *et al.*, 1983). In latitudes below 31ºS, however, it is only found in estuaries and is believed to complete its entire life cycle within these ecosystems (Potter *et al.*, 1986, 2015). This species was once the focus of a small commercial and iconic recreational fishery in the Swan-Canning Estuary in south-western Australia (Smith, 2006; Smithwick *et al.*, 2011), but sustained low abundances led to a reduction in commercial fishing effort and the eventual closure of that fishery in the 1970s, and greatly reduced recreational fishing effort since the late 1990s (Maher, 2002). Despite the overall reduction in fishing pressure on *M. dalli*, stocks in the Swan-Canning Estuary have not recovered and a restocking program was considered the best option to overcome the long-term recruitment failure by bypassing the high mortality rate that often occurs in the early larval stages (Smith *et al.*, 2007). The development of hatchery aquaculture techniques was initiated in 2012 and, to date, over 4.5 million post-larval prawns have been released (G. I. Jenkins, Australian Centre for Applied Aquaculture Research, Fremantle, Western Australia, unpublished data). While some information about the timing of reproduction and larval development in *M. dalli* is available (e.g., Potter *et al.*, 1986; Crisp *et al.*, 2016), detailed reproductive studies are required to ensure that the most mature broodstock are used, which maximizes hatchery production of larvae.

Understanding the reproductive biology of any penaeid species requires the description and quantification of oocyte development, known as oogenesis. Previous studies of oogenesis in penaeids often combined observations of somatic changes in ovarian morphology with histological changes in approximate proportion, size and number of oocyte types. Oogenesis of a number of species have been described including *Litopenaeus* (*Penaeus*) *setiferus* (Linnaeus, 1767) (King, 1948), *Fenneropenaeus (Penaeus*) *merguiensis* (De Man, 1888) (Tuma, 1967) and *Penaeus monodon* Fabricius, 1798 (Tan-Fermin & Pudadera, 1989). Such is the value of these descriptions that some have been applied to ecological studies of closely-related species *Melicertus* (*Penaeus*) *latisulcatus* (Kishinouye, 1896) (Penn, 1980), *Metapenaeus endeavouri* Schmitt, 1927, *Metapenaeus ensis*de Haan, 1844 (Courtney *et al.*, 1989), *Metapenaeus dobsoni* Miers, 1878 (De Croos *et al.*, 2011), and *Rimapenaeus* (*Trachypenaeus) similis* Smith, 1885 (Bauer & Lin, 1994), and in laboratory studies aimed at improving the reproductive potential of species such as *M. ensis* (Yano, 1985), *Farfantepenaeus paulensis* Pérez Farfante, 1967 (Peixoto *et al.*, 2005a), *Litopenaeus vannamei* Boone, 1931 (Palaclos *et al.*, 2003) and *Penaeus esculentus* Haswell, 1879 (Keys & Crocos, 2006). Recent histological observations of oogenesis in two species, *Metapenaeopsis dalei* Rathbun, 1902 (Sakaji *et al.*, 2000), and *Metapenaeus Monoceros* Fabricius, 1798 (Abraham & Manisseri, 2012), however, have shown marked differences in development of oocytes compared to the aforementioned studies, particularly in the formation of cortical bodies during the final maturation stage before spawning. This highlights the need for target-specific histological descriptions of oocyte size and composition during development of each species of interest.

Furthermore, histological observations of broodstock responses in penaeids, both to environmental parameters (Crocos & Kerr, 1983; Courtney & Masel, 1997; Cha *et al.*, 2004) and to laboratory experiments aimed to enhance production in domesticated aquaculture stock (Medina *et al.*, 1996; Bindhuja *et al.*, 2013), have been limited by their inability to quantify their effects on oogenesis. In the past, a combination of macroscopic and histological descriptions of oogenesis had been conducted by visual approximation only, such as those by Ayub & Ahmed (2002) and Peixoto *et*

al. (2003), but observations concurred with development in other penaeid species (see Dall *et al.*, 1990). For oogenesis to be precisely quantified, a microscopic method with replication at the cellular level is the only way to confirm changes in oocyte composition of gonads. Two key factors must be addressed for this to occur. Firstly, more precise methods of measuring the dimensions of oocyte cells must be found than is currently available and, secondly, these measures should be applied to a standardized method of quantifying the biovolume contributions of each oocyte cell type in a given area or volume within the gonad.

Cell size has traditionally been determined by taking single linear measures across a pre-determined axis at the equatorial plane with an ocular micrometer, with the equatorial plane defined as the largest possible two-dimensional area of a cell that included complete sections of nucleus in each cell (e.g., Peixoto *et al.*, 2005b). Precise measurements are nevertheless difficult to obtain using this method, due to the *in-situ* compression of the oocyte cells into non-geometric shapes. For example, inaccuracies have been shown in studies of several penaeids, *i.e., Metapenaeus affinis*Milne Edwards, 1837 (Ayub & Ahmed, 2002), *M. monoceros* (Abraham & Manisseri, 2012), and *F. merguiensis* (Tuma, 1967). New methods would therefore require the use of modern technologies to precisely quantify changes in oocyte size and composition during oogenesis. Solving these problems offers new opportunities to examine whether ovarian development in penaeids is consistent across different regions, fisheries and/or species and also in determining whether the effects of laboratory treatments to enhance reproductive condition are successful.

With this in mind, the aims of this study were to: (1) compare and contrast the ovarian development of *M. dalli* with other penaeids to verify its use as a model species by describing its morphological and histological changes during ovarian development, (2) use preserved histological samples of *M. dalli* ovaries to develop a more precise method for measuring oocyte dimensions and (3) further use preserved histological samples to develop a quantitative method of assessing the relationship between oogenesis and ovarian development in penaeids. Such novel methods should improve the precision of measures of size, and quantify the number and relative composition of oocyte cells during maturation

and subsequent recovery after spawning, allowing for more effective analysis of ovarian maturity in fisheries and aquaculture research.

Materials and methods

Collection of biological material

Female specimens of *M. dalli* were collected at night from the shallow waters of the Swan-Canning Estuary (31°56′50″S, 115°54′58″E) between December 2013 and March 2015, using a hand trawl net that was 1.5 m high, 4 m wide and constructed from 9 mm mesh. Upon collection, individuals were categorized using macroscopic observations into one of four stages of ovarian maturity, (immature, early maturing, late maturing and mature) on the basis of the descriptions in Ayub & Ahmed (2002). All prawns were chilled in an ice slurry until mortality, but not frozen to prevent potential damage to cells. A subset of those individuals whose gonads were classified as mature was first transferred to an aquaculture facility for spawning to demonstrate immediate effects on ovarian tissue of spawning/atresia, *i.e.,* the regression of acidophilic oocytes that have failed to be spawned, held under conditions modified from Laubier-Bonichon & Laubier (1976). Briefly, on arrival, the mature females were disinfected with a solution of 1 ppm formaldehyde for 30 min and placed in aerated holding tanks overnight (ASEAN, 1978). They were then stocked into 300 l conical base tanks with a flat mesh-lined floor at a density of up to 15 individuals per tank for 48 h. Tanks were filled with water with a salinity of 33‰ drawn from a bore accessing a saline aquifer, which was aerated constantly and maintained at a temperature of ~26° C with 0:24 h light: dark photoperiod. After spawning the females were transferred to an ice slurry as per the other individuals.

Morphology and histology

A subset of 25 individuals from each of the immature, early maturing, late maturing, and mature stages together with 25 post-spawned individuals from the aquaculture facility was examined in the laboratory. Each specimen was weighed to the nearest 10 mg (wet weight) using a Sartorius A200S

top balance and its carapace length, *i.e.,* orbital indent at the anterior end to the posterior edge of the carapace, measured to the nearest 0.1 mm using a Sontax 150 mm digital caliper. Ovarian tissue was then carefully excised and the Gonad Somatic Index (GSI) calculated using the equation GSI = (gonad weight / total weight) × 100. Subsamples of anterior or first abdominal sections of the excised gonads were immediately fixed before histological analysis for at least 48 h in a solution of tetraborate-buffered 10% formaldehyde with ~33‰ salinity.

The fixed gonad samples were then embedded into paraffin block and 6 µm sections taken as per Bell *et al.*, (1988). Sections were then stained with haematoxylin and eosin using the method adapted from Quintero & Gracia (1998). The resultant sections were then imaged using a Tucsen 9 MP camera mounted onto a compound microscope at 100 x magnification. The TSView 7 software package was used to download the images for analysis. Oocytes from these imaged samples were then characterized and compared to published histological descriptions (*i.e.,*Tuma, 1967; Ayub & Ahmed, 2002).

From these images, five oocytes of each type per slide were selected *ad-hoc* from 10 haphazardly selected slides and measured via two methods with ImageJ 1.48 64-bit software, producing 50 measures of each cell type overall for each method. The first method was the traditional single linear measure to determine diameter (Ayub & Ahmed, 2002). Diameter was measured parallel to the long axis of each mounted slide. The second method was the measurement of the external circumference by tracing the perimeter of each cell. Both measurements were taken as close to the equatorial plane as possible, with this plane being defined by those oocytes that showed the largest possible two-dimensional surface area and included complete sections of nucleus within each cell. In cortical oocytes where the nucleus is not visible, this was defined as the largest possible area of cytoplasm that could be found with cortical bodies of uniform size and shape around the periphery.

Measurements were made at the equatorial plane to ensure that only sections of whole cells were used. Partial sections of cells are often created as oocytes are contorted into non-geometric shapes by being compressed in the ovary. Each measurement was made on a digital image at 100× magnification then re-calibrated to µm using an image of a micrometer at the same magnification.

Once perimeter measures were made, equivalent diameter and spherical biovolume were calculated using the following equations, assuming that oocytes released from gonads take on a spherical form.

$$\text{Diameter (sphere)} = \text{circumference} / \pi$$

$$\text{Volume (sphere)} = (4/3) \times \pi \times r^3, \text{ where } r = \text{diameter} /2$$

The diameters measured or calculated using the traditional linear measure and new perimeter method were compared to determine which technique provided the most consistent results, as demonstrated by the size of the 95% confidence limits.

Oocyte development

The oocyte types present in each developmental stage were described visually from the images taken from the slides, as per traditional methods (*i.e.,*Tuma, 1967; Ayub & Ahmed, 2002). The relationship between the relative proportions of each oocyte type present and the stages of development were then examined to determine trends in oocyte development between stages.

Images of each slide were uploaded to Adobe Photoshop CS6, where 400 × 400 µm gridlines were applied. These dimensions were chosen on the basis that at least six replicate grids could be obtained from each slide for analysis. From this, three grid squares were haphazardly selected to enumerate each cell type over the two-dimensional plane. This involved counting the number of oocytes of each cell type within each square, taking care to only include cells in, or near, to the equatorial plane. Cells that overlapped grid boundaries were only considered when crossing the left or top sides of the nominated grid square(s), as adapted from the standard haemocytometer method of counting cells (ASEAN, 1978).

Once each oocyte type was enumerated, the total biovolume for that type of oocyte in each replicate was calculated by multiplying the number of that oocyte type by its corresponding mean biovolume (see equation below). This was done to standardize the biovolume of each cell type in each replicate. Once calculated, the proportions of each oocyte type at each stage were compared and contrasted to determine the relationship between relative oocyte abundance and macroscopic stage of development.

Total biovolume (oocyte type) = mean biovolume

(oocyte type) n (oocyte type)

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine whether carapace length and GSI differed among various stages of ovarian development. Analysis of the linear relationship between the $\log_e$ mean and $\log_e$ standard deviation of each data set was used to determine which transformations, if any, were required, to meet the test assumption of homogeneity of variance for each of the above two data tests (Clarke *et al.*, 2014a). This analysis indicated that a square root transformation of GSI values were required, while carapace length required no transformation. When ANOVA detected a significant difference, *post-hoc* tests were conducted using Tukey's HSD to elucidate the pairs of ovarian stages that were responsible for each of those differences. In this and all tests, a null hypothesis of no significant difference between *a priori* groups was rejected when P was < 0.05.

To determine which of the two methods for calculating cellular diameter (see above) was the most precise, the data for each of the five oocyte types was analyzed using multiple pairwise comparisons, via two-tailed *F*-tests. If a significant difference in magnitude of variance was detected, the magnitude of that difference was quantified by calculating the percentage change in variance from the single linear to the new perimeter method. All univariate analyses were performed using SPSS version 22 software (IBM, 2013).

Multivariate analysis to determine whether the percentage contributions of the biovolumes of each type of oocyte present (*i.e.,* oocyte composition) differed among ovarian development stages was conducted using a one-way Analysis of Similarities (ANOSIM; Clarke & Green, 1988) test using the PRIMER v7 software package (Clarke & Gorley, 2015). Prior to analysis, the percentage contributions of the biovolumes of each cell type from each section were visualized using shade plots and an appropriate transformation selected using the criteria in Clarke *et al.*, (2014b). In this case, a square-root transformation was used to avoid any tendency for a cell type to be excessively dominant. This pre-treated data was used to produce a Bray-Curtis resemblance matrix, which was, in turn,

subjected to the one-way ANOSIM test. To visualize the patterns exhibited by oocyte type among the 125 replicate samples, a non-metric multidimensional scaling (nMDS) ordination plot (Clarke, 1993) was constructed from the above resemblance matrix. To simplify and further illustrate the histological differences between stages, a second (centroid) nMDS plot was produced using a distances among centroids matrix, which creates averages in the 'Bray-Curtis space' from the 25 replicate samples representing each stage (Lek *et al.*, 2011). Stacked bar histograms, representing the untransformed percentage contributions of the various cell types were included to indicate which types of oocytes were responsible for the changes in cellular composition during each stage of oogenesis.

Results

Macroscopic observations

The gonad *in situ*, from the dorsal carapace to the dorsoventral section of the tail above the anus showed macroscopic changes between each of the five stages.

- Stage 1 (immature). Gonad not visible through the exoskeleton, requiring dissection to observe. Gonad translucent in appearance and smooth textured, with the diameter in the mid-section smaller than the intestinal tract directly below. Anterior lobes present, although undeveloped.
- Stage 2 (early maturing). Gonad visible through dorsal exoskeleton. Upon dissection gonad appears thin with a white-yellow or green-yellow granular appearance. Anterior lobes enlarged and extend forward into the carapace, while the posterior lobes greater in size than the intestinal tract.
- Stage 3 (late maturing). Gonad clearly visible through the exoskeleton, taking on a green granular appearance. Texture of the dissected gonad firmer and lobes nearly filled out. Anterior lobes fully-formed, but do not fill the carapace completely. An 'arrow head' shape begins to form posteriorly in the final abdominal segment above the anus.

- Stage 4 (mature). Gonad now clearly visible through the exoskeleton, expanding in size to occupy much of the carapace and a significant portion of the abdominal region. Upon dissection has a dark-green or green-brown granular appearance and a distinct 'arrow head' formed in the posterior end.
- Stage 5 (post spawning). Gonad either slightly visible or no longer visible through the exoskeleton. Upon dissection, gonad appears opaque-white or white-yellow and smooth in texture. A red hue present in some samples.

Carapace length and gonad somatic index

Carapace lengths were shown by one-way ANOVA to differ significantly among stages of development ($F = 11.84$, $df = 4, 124$, $P < 0.001$), with Tukey's post hoc test determining that immature and early maturing females had a smaller mean carapace length (~ 19 mm) than the late maturing, mature and post-spawned females (~ 21–22 mm; Fig. 1). Overlap in 95% confidence limits of some of the stages of ovarian development indicates that it is not well defined by carapace length. A one-way ANOVA of the GSI values demonstrated that they differed significantly among the stages of development ($F = 250.8$, $df = 4, 124$, $P < 0.001$), with Tukey's *post-hoc* test demonstrating that significant differences existed between all stages, except between early maturing and post spawning (Fig. 2). The mean GSI values increased sequentially from 0.58 when immature to a maximum of 6.90 when mature, followed by a sharp decrease at post spawning to 1.97.

Observations of oocyte appearance

Preliminary observations indicated that the arrangement of oocyte cells varied greatly between individuals within each stage of development, with the *in situ* observations of development indicating that oocyte cells migrate from the germinal zone (Fig. 3a) to the periphery of the ovary during development. Cell types such as yolky and cortical oocytes later become bound by follicle cells just prior to spawning. Using the criteria defined by Ayub & Ahmed (2002), five types of oocytes (chromatin nucleolar, perinucleolar, yolkless, yolky, and cortical), were identified in addition to follicle cells in ovarian sections stained in haematoxylin and eosin. These cells showed a progression

in size, with earlier stages comprising primarily basophilic cellular material, staining blue, tending to shift to acidophilic cellular material, staining red, during the later stages of ovarian development. Oocyte cells migrate away from the germinal zone (Fig. 3a) as they develop from chromatin nucleolar to cortical oocytes, leaving room for new cells to be produced. The appearance of each oocyte type is described below.

Chromatin nucleolar oocyte (Fig. 3a). Constructed primarily of a densely basophilic nucleus, containing chromatin material in no particular arrangement. Cell has very little cytoplasm that is completely basophilic.

Perinucleolar oocyte (Fig. 3a). Exhibits a densely basophilic nucleus with a larger basophilic cytoplasm. Basophilic nucleoli are arranged around the periphery of the nuclear membrane. Follicle cells appear to arrange themselves around the outside of some perinucleolar oocytes (Fig. 3a).

Yolkless oocyte (Fig. 3b). Cytoplasm now clearly acidophilic, with nuclear membrane clearly defined.

Yolky oocyte (Fig. 3c). Cytoplasm now exhibits acidophilic yolky ‘plates’ or granules that include cytoplasmic vesicles and/or cortical crypts. Chromatin material and nucleoli are much greater in number than the yolkless oocyte, resulting in the nuclear membrane becoming denser and less distinguishable. Each yolky oocyte has a layer of follicle cells surrounding each cell.

Cortical oocytes (Fig. 3d). Nucleus appears absent or near absent. Cortical cells contain small oval-shaped cortical bodies arranged on the internal periphery of the cytoplasm at the cell membrane. These bodies are a defining characteristic of the mature stage of the oocytes.

Atreatic oocytes (Fig. 3e). Appear as remnants of acidophilic oocytes that failed to spawn, containing no cytoplasmic material or a nucleus.

Oocyte size and composition

Pairwise comparisons of the size of each of the five types of oocyte measured using the single linear or perimeter method, conducted using two-tailed *F*-tests, demonstrated that in all cases there was a

significant difference in the variance (Table 1). The perimeter method resulted in a 17 to 40% reduction in the 95% confidence limits of the calculated diameter of the cell, depending on the type of oocyte.

One-way ANOSIM detected a significant difference (Global $R = 0.787$, $P = 0.001$) in oocyte composition, with pairwise comparisons indicating that the contributions of the various cell types to each ovarian developmental stage were different in all stages, except between immature and post spawning (Table 2). This is illustrated on the nMDS plot where the points representing the gonads from immature and post spawning stages overlap considerably and are well separated from those points representing the other stages, which all form discrete groups (Fig. 4a). The centroid nMDS plot illustrates the clockwise progression in oocyte composition (Fig. 4b), with immature and post-spawning ovaries comprised solely of chromatin and perinucleolar oocytes (Fig. 5). Although early- and late-maturing ovaries retained chromatin and perinucleolar oocytes, they were characterized by the presence of a substantial proportion of yolkless oocytes, with late-maturing gonads also containing yolky oocytes and a low proportion of cortical oocytes. By the mature stage, the ovary is comprised almost exclusively of yolky and cortical oocytes. Following spawning, these yolky and cortical oocytes are expelled or re-absorbed, leaving chromatin and perinucleolar oocytes. Thus, oocyte compositions in immature and post spawning ovaries are almost identical.

Discussion

Macroscopic observations

Macroscopic and histological observations of the gonads of wild-caught female *M. dalli* showed that the oogenesis was separated into five distinct developmental stages, progressing from immature, to early maturing, late maturing, mature and finally post spawning. This is similar to previous works on *L. setiferus* (King, 1948), *F. merguiensis* (Tuma, 1967), *Farfantepenaeus (Penaeus) brasiliensis*Latreille, 1817 (Quintero & Gracia, 1998), and *M. affinis* (Ayub & Ahmed, 2002). Macroscopic differentiation of maturity in *M. dalli* was possible for early maturing, late

maturing and mature stages of ovarian development; however, the gonad of immature and post-spawned individuals could not be distinguished from each other. The lack of differences in macroscopic observations for these two stages was due to the fact that the exoskeleton obscured the view of the gonad *in situ*, and that each gonad was relatively similar in size and colour. Differentiation of these stages was definitive only when gonad was excised and histological analysis was performed, with gonads taken from spawned individuals containing significant amounts of atreatic cells and extraneous material. Late maturing and mature gonads were considerably easier to distinguish externally through the exoskeleton in *M. dalli* than in *F. brasiliensis* (Quintero & Gracia, 1998), but are most consistent with findings from *F. merguiensis* (Tuma, 1967) and *M. affinis* (Ayub & Ahmed, 2002).

Carapace length and gonad somatic index

Strong significant differences were observed in the GSI between stages of ovarian development in this study ($P < 0.001$), with GSI increasing sequentially from a minimum when immature, to a maximum when mature, before declining after spawning. The mean GSI of the mature stage in this study (6.9 ± 0.6) was similar to that recorded in the neighboring Peel-Harvey Estuary (7.0 ± 0.4) during the 1987–88 breeding season. The far greater *F*-value for GSI (250.8) than carapace length (11.84) indicates that GSI provides a more precise measure of ovarian developmental stage. Although it was possible to differentiate overall ovarian development based on carapace length ($P < 0.001$), *post hoc* analysis indicated that the five ovarian developmental stages formed only two significantly different groups, *i.e.,* immature and early maturing *vs* late maturing to post spawning. This provides relatively poor discrimination between stages of ovarian development.

The increase in carapace length with ovarian developmental stage is due to the fact that the breeding of *M. dalli* occurs during the warmer summer period (October-March), which coincides with a significant increase in somatic growth rates (Potter *et al.*, 1986, 1989). Carapace lengths of mature *M. dalli* in this study (22.3 mm) closely matched those of female *Metapenaeus bennettae*Racek & Dall, 1965 (22.4 mm) in Moreton Bay, Queensland (Courtney & Masel, 1997). It is noteworthy that the range of carapace lengths between late maturing to post spawning female *M. dalli* in this study (20.6–

22.3 mm) are larger than corresponding values recorded in the neighboring Peel-Harvey Estuary in 1987–88 (17.9–20.7 mm; Potter *et al.*, 1989). This demonstrates that although the GSI of mature females is similar between these two systems, the size of individual females is greater in the Swan-Canning Estuary.

Oocyte size and development

During ovarian development, distinct changes were observed in oocyte size and composition from the immature stage, through early and late maturation to the mature stage. At post spawning, the ovary was similar in appearance to that of the immature stage; however, the post-spawned ovary could be distinguished by the presence of large deposits of non-oocyte material and atreatic oocytes. While the changes in oocyte composition are consistent with those observed in *M. affinis* (Ayub & Ahmed, 2002), they differ from those of *M. monoceros* (Abraham & Manisseri, 2012) and *M. dalei* (Sakaji *et al.*, 2000). This is due to the last two species containing oocytes without cortical bodies at the mature stage.

Measurements of oocyte diameter using the new perimeter method were found to reduce the range of the 95% confidence limits by between 17–40% for each of the five oocyte types when compared to those calculated using the single linear method. This increased level of precision is particularly useful when quantifying the effects of spatial, temporal, nutritional, and environmental changes on ovarian conditioning in the field and/or in the laboratory. The method developed in the current study would have enhanced findings by Rao (1973), who found considerable differences in oocyte size from wild caught *M. dobsoni*, *P. indicus*, and *P. stylifera* from Cochin when compared to other parts of India. Similarly, findings by Ayub & Ahmed (2002), which compared oocyte sizes in *M. affinis*, *P. indicus*, and *P. stylifera* from coastal waters off Pakistan, with similar environments in India, would have been more precise. In laboratory studies for aquaculture purposes, this quantitative method of assessing ovarian development can be applied to studies exploring the effects of altering food composition, physiology and/or rearing conditions in broodstock domestication. Quantitative analysis of these treatments on ovarian development in *Melicertus* (*Penaeus*) *kerathurus* (Forskål, 1775) (Medina *et al.*, 1996), *F. paulensis* (Peixoto *et al.*, 2005b), *P. esculentus* (Keys & Crocos, 2006), and *P.*

monodon (Marsden *et al.*, 2007) would have significantly enhanced the qualitative histological comparisons that were made.

Ovarian oocyte composition

Relationships between oocyte composition and ovarian development in this study indicated that the greatest diversity in oocyte cells existed during proliferation phase of the early and late maturing stages, with cortical oocytes making up the majority of cells present in the mature stage. This phenomenon has been described in several other studies and by Dall *et al.* (1990), but without any statistical analysis to support these claims. This study is therefore the first to statistically demonstrate changes in oocyte composition with each developmental stage. Given the nature of the pairwise comparisons between stages analyzed histologically, and the absence of chromatin, perinucleolar and yolkless oocytes in mature gonads, it could be assumed that the final maturation process is rapid. Changes in GSI between late maturing and mature stages also support this assertion, indicating that much of the energetic and nutritional reserves during this period are bestowed to the oocytes, particularly lipids (Cahu *et al.*, 1994), α-tocopherol, and ascorbic acid (Cahu *et al.*, 1995). Post-spawning absorption of atreatic oocytes and extraneous material in the gonad may act as a recovery mechanism to stave off mortality after spawning, allowing for recovery and repeated spawning, but additional environmental stressors may increase mortality during this sensitive period. The novel method of determining the size of oocytes in the ovaries of penaeids developed in this study showed a much greater precision than the traditional techniques previously used. Having using these measures, multivariate statistical analyses were employed to describe the relationship between oocyte composition and ovarian development, resulting in the first quantifiable data to be obtained by any study of oogenesis for a penaeid. New opportunities now arise in the application of these methods in studies of the ovarian development in penaeids, with practical applications in assessing the reproductive performance of wild-caught female prawns and those held under the influence of alimentation, relatively different to what would be found in natural environment.

Disclosure statement

No potential conflict of interest was found by the authors.

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Figure 1. Mean carapace length of female *Metapenaeus dalli* at each of the five stages of ovarian development. Error bars represent 95% confidence limits and the letters indicate significant differences among stages as determined by Tukey's HSD test.

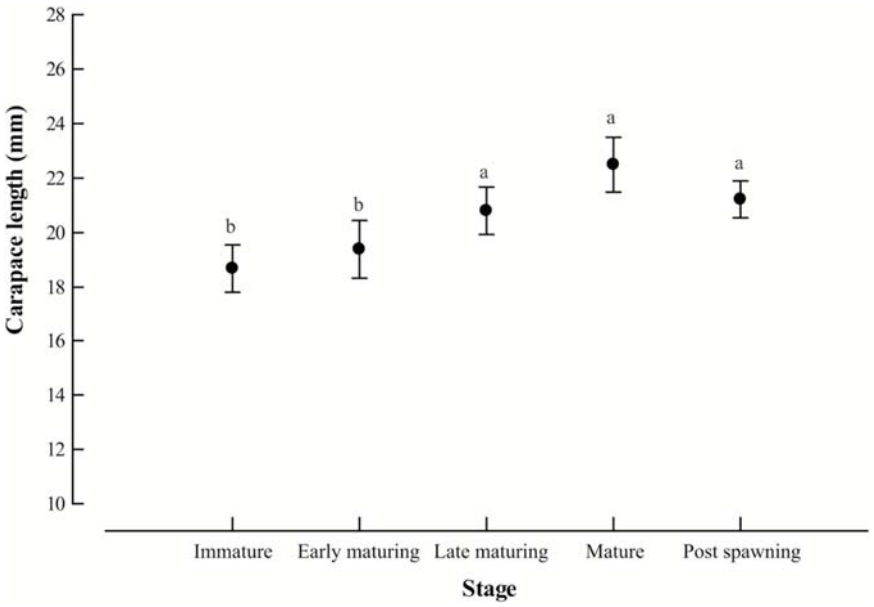

Figure 2. Mean Gonad Somatic Index (GSI) values for female *Metapenaeus dalli* at each of the five stages of ovarian development. Error bars represent 95% confidence limits and the letters indicate significant differences among stages as determined by Tukey's HSD test.

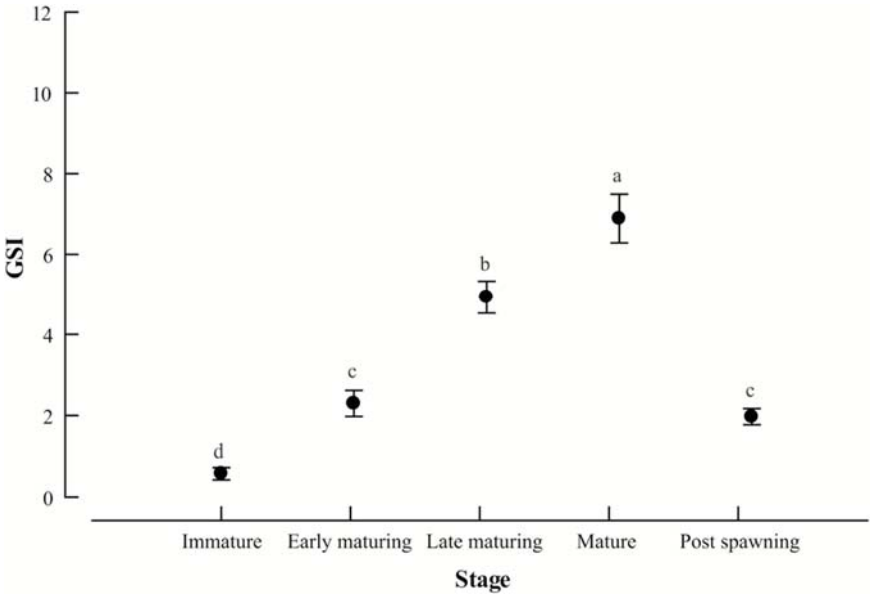

Figure 3. Representative photographs of haematoxylin and eosin stained oocytes of (**A**) immature, (**B**) early maturing, (**C**) late maturing, (D) mature, and (**E**) post-spawning female *Metapenaeus dalli* at 250× magnification. Images of cells were taken at the ovarian development stage at which they first appear. FC, follicle cells; CR, chromatin nucleolar oocytes; PO, perinucleolar oocytes; GZ, germinal zone; N, nucleus; N', nucleoli; VES, cytoplasmic vesicles; CO, cortical bodies; AT, atreatic cell. Bar = 50μm.

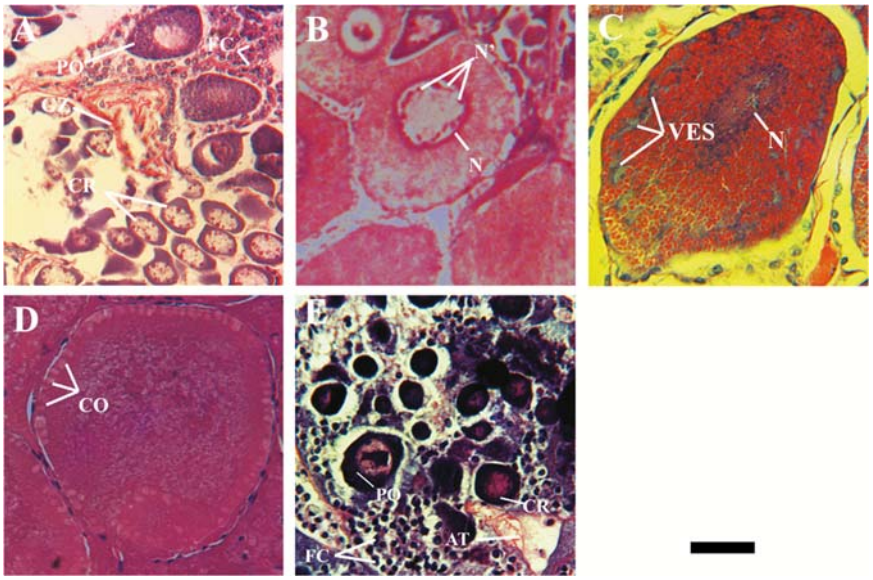

Figure 4. **A.** Non-metric multidimensional scaling (nMDS) ordination plot derived from a Bray-Curtis resemblance matrix of the square-root transformed percentage biovolumes of each type of oocyte from 25 female *Metapenaeus dalli* from each of the five stages of ovarian development. **B.** Centroid nMDS ordination plot, derived from distance among centroid matrices constructed from the above Bray–Curtis resemblance matrix. Arrows on dotted lines indicate the progression in development of ovaries from immature to post spawning.

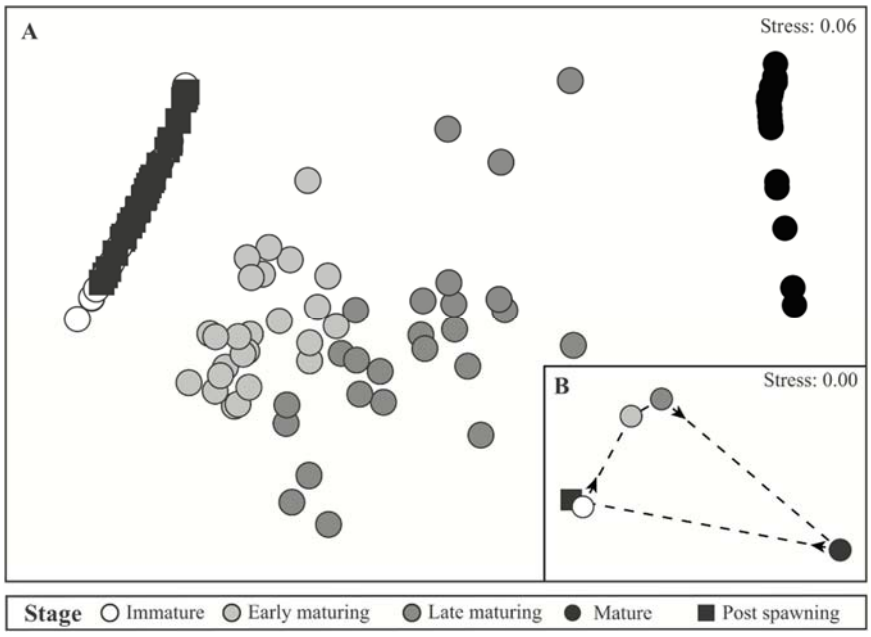

Figure 5. Mean percentage contributions of the biovolumes of each type of oocyte in female *Metapenaeus dalli* from each of the five stages of ovarian development.

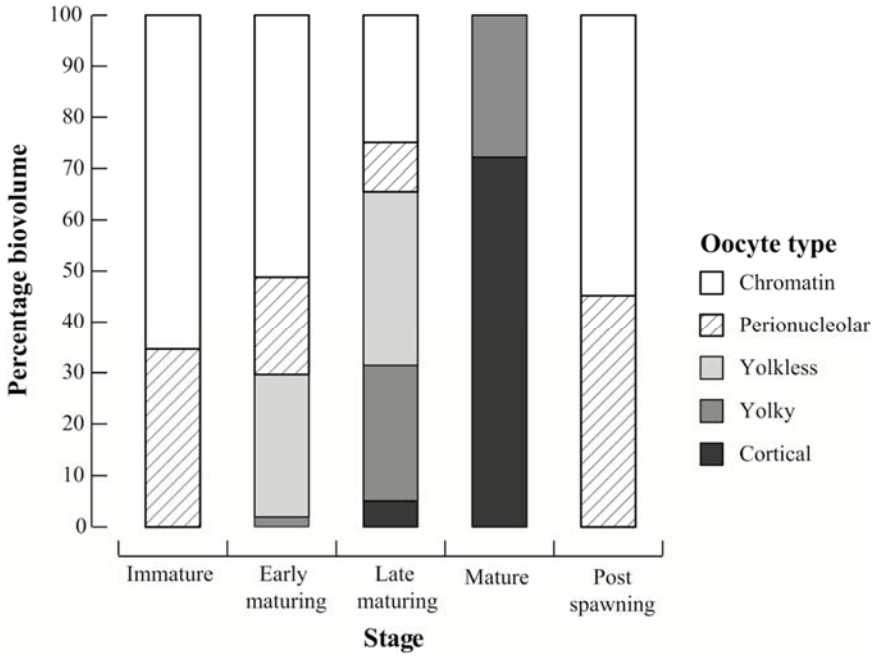

Table 1. Mean diameters (μm) and ± 95% confidence limits of five types of oocyte present in the ovaries of female *Metapenaeus dalli* during ovarian development, calculated using single linear and perimeter methods. The significance values (*P*) of *F*-tests are provided together with the percentage reduction in confidence limits achieved using the perimeter method.

Oocyte type	**Linear measure**		**Perimeter measure**	***P***		**% reduction**
Chromatin	26.59	± 1.78	23.32	± 1.48	0.001	17
Perinucleolar	55.46	± 4.41	60.63	± 2.96	0.000	33
Yolkless	94.94	± 6.31	102.69	± 3.79	0.003	40
Yolky	128.84	± 6.95	131.79	± 4.48	0.001	35
Cortical	143.05	± 7.98	152.55	± 5.55	0.000	30

Table 2. Pairwise *R* statistic and significance level (*P*) values derived from a one-way ANOSIM of the square-root transformed percentage biovolumes of each type of oocyte from female *Metapenaeus dalli* in each of the five stages of ovarian development. Insignificant pairwise comparisons ($P > 0.05$) are shaded grey.

	Immature	**Early maturing**	**Late maturing**	**Mature**
Early maturing	0.816			
Late maturing	0.901	0.427		
Mature	1.000	1.000	0.934	
Post spawning	0.018	0.833	0.903	1.000

This is a non-significant and the comparison cell of Immature-Post spawning background needs to be shaded in grey.