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Growth pattern, diet and reproductive biology of the clownfish *Amphiprion ocellaris* in waters of Pulau Tioman, Malaysia

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ABSTRACT

The growth pattern, diet and reproductive biology of the clownfish *Amphiprion ocellaris* collected from waters of Pulau Tioman were investigated. The length-weight relationship showed an isometric growth pattern (b = 3) in *A. ocellaris*. The stomach contents mainly consisted of zooplankton and algae, which showed that the fish is omnivorous and was confirmed by trophic level analysis (2.98 ± 0.29). Fecundity of *A. ocellaris* ranged from 23-1518 with mean egg count of 582 ± 478, and has positive relationships with body length, body weight, eviscerated weight and ovary weight. The gonads were also described and examined histologically. The ovaries showed 4 stages of maturity, displaying different colours for each stage. The different developments of oocytes were also found in each stage of maturity. The males and non-breeders comprised of both testicular and ovarian tissues. In the males, the testicular tissues were more prominent in the ovotestes, whereas in non-breeders the primary oocytes were more prominent. Absence of testicular tissue in ovaries of females showed that the fish is a protandrous hermaphrodite and sex change may not be reversible. Similar observations have been reported in *A. ocellaris* of other countries and other *Amphiprion* species indicating that *Amphiprion* species show consistency in their reproduction strategy throughout their range.

Introduction

Malaysia initially focused on the export of freshwater species, but now is one of the main suppliers of marine ornamental species which are becoming increasingly popular among aquarist (Abol-Munafi et al., 2011; Cato and Brown, 2003). The false clownfish, *Amphiprion ocellaris*, is one of the most commercially exploited species in the aquarium trading as ornamental fishes (Wabnitz et al., 2003). It is unfortunate though that in the present situation with increasing demand for the clownfishes, supplies are mostly dependant on the wild catches (Abol-Munafi et al., 2011; Cato and Brown, 2003). Studies on Malaysian pomacentrids are still lacking (Sin et al., 1994), and concerns have been raised about the declining numbers of this species in Malaysian waters due to the possibility of overfishing and deterioration of its natural habitat caused by destructive collection methods (Abol-Munafi et al., 2011; Livengood and Chapman, 2007). Therefore, it is important to be able to propagate this species in captivity for both commercial and conservation purposes. Although the clownfish *Amphiprion ocellaris* has been successfully reared in captivity in other countries (Juhl, 1992; Moe, 1992), the production in Malaysia is still low (Abol-Munafi et al., 2011). In Malaysia, Liew et al. (2006) has successfully bred the clownfish in captivity but failed to develop broodstocks from F1 generations and still needs to collect broodstocks pairs from the wild.

Amphiprion ocellaris are protandrous hermaphrodites; they have the ability to change sex from male to female at maturity (Thresher, 1984; Fautin and Allen, 1992). Sex change occurs in relation to the social hierarchy, where the two largest individuals (female being the largest of the two) forms a strong monogamous breeding pair while the rest are non-breeders (Allen, 1975; Moyer and Nakazono, 1978; Thresher, 1984; Fautin and Allen, 1992; Hirose, 1995; Liew et al., 2006). When the female dies (or is removed), the male changes sex to become the dominant breeding female and the second largest member from the non-breeders becomes the dominant male (Rosenberg and Cruz, 1988; Fautin and Allen, 1992). This has made the breeding of clownfish

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challenging in captivity; the need for research arose from the lack of detailed information concerning the feeding and reproductive biology of *A. ocellaris* in Malaysian waters, which are important to facilitate effective management in culturing the species to provide for local and global demands. Therefore, this study was conducted to measure important biological aspects of *A. ocellaris*; namely (i) population growth pattern (ii) stomach content analysis (iii) trophic level (iv) fecundity estimates, (v) observations of changes in maturity stages in the gonads and, (vi) histology studies to determine the stages of the reproductive organ.

Material and methods

Fish sampling

A total of 65 fishes were collected from waters of Pulau Tioman using plastic bags while SCUBA diving. The fishes were then killed by a sharp blow on the head or by pithing. The specimens were then measured to the nearest 1 cm (total length TL and standard length SL) and weighed to the nearest 0.1 g (total body weight TW and eviscerated weight EW). Each fish was dissected, the gonads were removed, weighed and photographed.

Length-weight relationship

Population growth pattern was estimated based on length-weight relationship (LWR), expressed by the equation $W = aL^b$ where W is body weight (g), L is total length (cm), a is the intercept and b is the slope. A non-linear regression was used to determine the values of a and b. Curve fitting was carried out by a non-linear iterative method using Levenberg-Marquardt and simplex logarithm using MicroCal Origin™ Version 8.0 programme with a statistical significance of 5%. The degree of adjustment of the model studied was assessed by the coefficient of determination (r^2). A student t-test was applied to verify if the b value presented a significant difference to 3.0, indicating the type of growth; isometric (b = 3.0), positive allometric (b > 3.0) and negative allometry (b < 3.0) (Froese 2006; Simon and Mazlan, 2008; Simon et al. 2009; Froese et al., 2011, 2014; Simon et al. 2014).

Stomach content analysis and trophic level

In the laboratory, the digestive tracts were removed and their content analysed under a microscope and identified. Diet composition data were used to estimate the trophic level of the fish. TROPH value was calculated from the dataset using TrophLab; an application for estimating TROPH and its standard error using weight or volumetric contribution and trophic level of each prey species to the diet, where individuals with a value of 2.0 or lower were considered herbivores, 5.0 and above were carnivores and any value in between those values were omnivores (Pauly et al., 1998, 2001; Simon and Mazlan, 2010).

Fecundity estimates

Fecundity was estimated from 30 females (n = 30) as the total number of oocytes present in the mature gonad. Prepared samples were fixed in Gilson's fluid to loosen the oocytes from the lobe. The total number of oocytes were then counted and reported as total fecundity. Relationships between fecundity and total length, total weight, ovary weight and eviscerated weight were then derived by regression analysis using MicroCal Origin™ software and were analysed using Pearson's correlation coefficient (Simon et al. 2012).

Gonad histology

A total of 30 gonads from all development stages were prepared and placed in cassettes, dehydrated (tissue processing) and embedded in paraffin wax following the procedures as practiced by the National Fish Health Research Centre of Fisheries Research Institute laboratory. Sections were cut at 5μm thickness and then dewaxed, dried and stained with hematoxylin and eosin. They were then mounted on slides before being observed under the microscope (Leica DM1000) equipped with a camera. Diameter of oocytes of all stages were randomly selected and measured to the nearest 0.001mm.

Results

Length-weight relationship

Length-weight relationship was derived from 65 individuals of clownfishes. The length weight relationship was a combination for both sexes and was represented as $W = 0.0197L^{3.06}$ (Fig. 1). Intercept 'a' was 0.0197 ± 0.0037 and the value of exponent 'b' was 3.0674 ± 0.0917, with coefficient of determination 'r^2' value of 0.9429 ($P < 0.001$). The value 'b' was in close proximity of 3, indicating an isometric growth pattern where the body changes form in proportionate to the body weight (following cube law; volume = L^3).

Stomach content and trophic level

The stomachs of 60 fishes were examined, most of which were empty or contained few food item. Those food items were identified to mainly consist of various larvae (barnacle, tunicate, crustaceans and gastropods), copepods, algae, fish eggs and ctenoid scales (Fig. 2). The estimated trophic level ranged from 2.00 to 4.10 with mean values of 2.98 ± 0.29, and remained at the same level as size increased (Fig. 3a and b).

Fecundity estimates

Total fecundity ranged from 23-1516 eggs with a mean egg count of 582±478. An overall positive correlation was obtained with fecundity and each and every comparison including body length (TL), body weight (TW), body eviscerated weight (EW) and ovary weight (Fig. 4) with r values of 0.308, 0.223, 0.154,

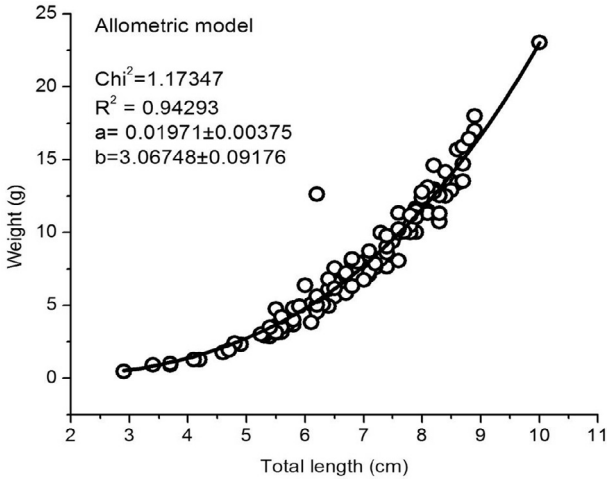

Fig. 1. Length weight relationship of *A. ocellaris* from Pulau Tioman waters. Red line represents a non-linear fit of the *A. ocellaris*, both sexes combined.

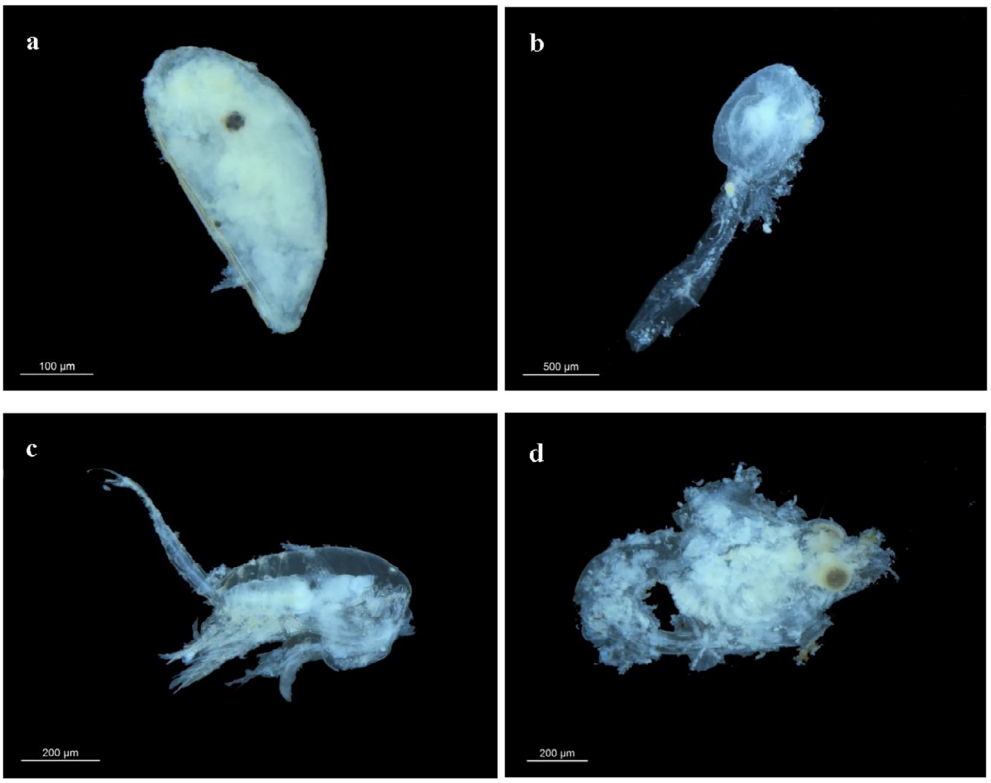

Fig. 2. Major food items consumed by *A. ocellaris* in Pulau Tioman. (a) barnacle larvae (b) tunicate larvae (c) copepod and (d) zoea larvae of crustaceans

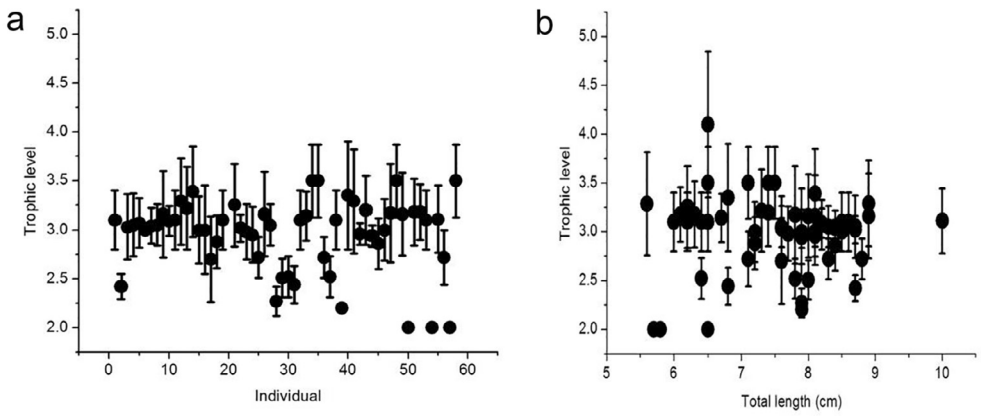

Fig. 3. Trophic level of *A. ocellaris*. (a) Identification of trophic levels of *A. ocellaris* individuals. (b) Identification of trophic levels of *A. ocellaris* according to size. Open circles represent mean TROPH values/trophic level and solid bars represent variants of prey items.

and 0.660 respectively. Fecundity showed a strong correlation with ovary weight (r = 0.660) but a weaker correlation with body length (r = 0.308).

Gonad development in A. ocellaris

The gonads of *A. ocellaris* are bi-lobed and asymmetrical (Fig. 5e and 6e). Ovotestes are found in males and non-breeders. The shape of the ovotestis is flat compared to the ovary and is translucent white in colour. Histological examinations revealed that the ovotestes in male and non-breeders consist of both testicular and ovarian cells. Ovotestes of male fishes comprised all stages of spermatogonic cells (spermatogonia, spermaticytes, spermatids and spermatozoa) and are located in the centre region (TR), surrounded by oogonia with pre-vitellogenic oocytes at the periphery (OR) (Fig. 5a and b). Ovotestes of non-breeders consist mainly of oogonia and previtellogenic oocytes (OR) , with less area of the testicular region (TR) although all stages of spermatogenic cells can be found (Fig. 5c and d). The heterosexual germinal elements are in direct contact with each other with no connective tissues in both ovotestes.

As for the ovary, testicular tissues are not observed in immature and mature females, where only mixtures of all stages of oocytes are present (Fig. 5e and f). Four developmental stages were recognised according to major morphological characteristics of oocytes growth. Terminology used for categorising oocytes based on the microscopic and macroscopic features of the ovaries were adapted from Abol-Munafi et al. (2011), McMillan (2007), Genten et al. (2009), Wallace and Selman (1981) and Selman and Wallace (1989).

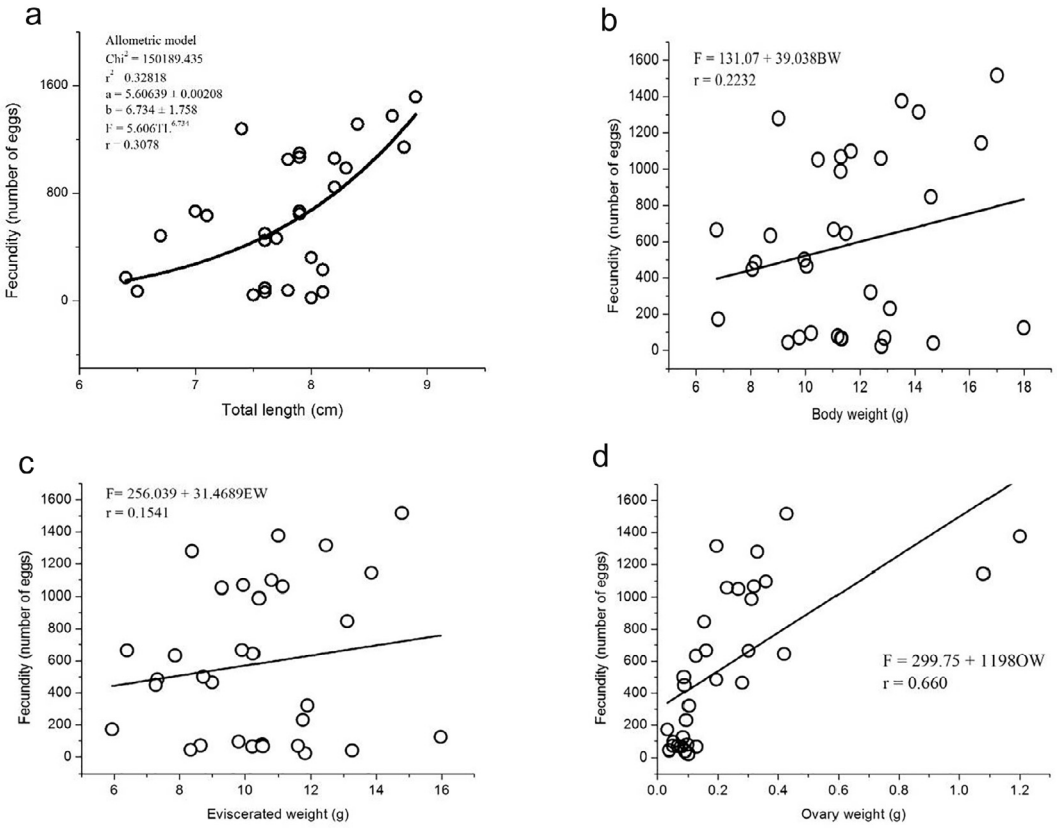

Fig. 4. Relationship between fecundity and a) body weight b) total length c) eviscerated weight and d) ovary weight in *A. ocellaris*. Solid line represents a non linear fit in (a) and linear fit in (b-d) of *A. ocellaris*

Stage I ovaries are small in size, colour ranging from white to pale yellow (Fig. 6e). Primary growth oocytes are predominant (Fig. 6a). Oogonia are small (54.03±1.2μm) with the presence of one nucleolus and are highly basophilic. The nuclei may contain many prominent nucleoli, often at the periphery of the nucleus. Oogonia initiated meiosis and entered into chromatin nucleolus stage of development. They are considered oocytes in this stage. Oocytes are surrounded by a single layer of squamous follicle cells (zona granulosa).

The colour deepens as ovaries turns from pale to bright yellow in Stage II (Fig. 6e). This is the beginning of secondary growth with cortical alveoli (CA). Vacuoles start to appear and progressively increase in number to form peripheral rows (Fig. 6b). Oocytes are 167.11±18.52μm in size. This can be found in premature females.

In Stage III, the ovaries are matured and are bright orange in colour (Fig. 6e). The appearance of yolk granules (protein) and fat vacuoles in the cytoplasm defines the vitellogenic oocytes (Vtg). The cytoplasm begins to fill with yolk spheres, granules or globules (Fig. 6c). These structures maintained their shape throughout the growth without merge into a mass of yolk fluid, with an average size of 239.23 ± 23.24μm. This stage is predominant in mature female ovaries.

Stage IV ovaries range from dark yellow to brownish (Fig. 6e). The germinal vesicle begins to breakdown and migrates towards the animal pole with the dissolution of the nucleolus membrane (Fig. 6d). Rapid uptake of fluid by the oocytes (hydration) causes the oocytes to swell and ovulation occurs when the follicle ruptures after the completion of oocyte maturation. The oocytes in this stage measured an average of 280.25 ± 17.52 μm in size. The remnants follicles are called postovulatory follicles.

Discussion

In the current study, most of the stomachs were empty due to regurgitation of the fish during capture process. Therefore, stomach fullness analysis was unable to be carried out and thus, the diet composition analysis was based on the remaining stomach content. The contents found were similar to the reports by Myers (1999) and Nakamura et al. (2003). A number of stomachs contained fish eggs and scales, suspected to be the result of egg guarding behaviour of the clownfish.

The results showed that *A. ocellaris* exhibited an isometric growth pattern where the length increased proportionately to weight, similarly Froese et al. (2014) reported matching value in the estimation of parameter 'b' for the majority of *Amphiprion* members; 3.04 for *A. percula*, *A. melanopus* and *A. akallopisos* and 2.99 in *A. perideraion*, *A. polymnus* and *A. clarkii*, indicating that the members of the genus may all show the same isometric growth strategy.

The fecundity estimates of *A. ocellaris* were similar as reported by Fautin and Allen (1992) and Gordon and Bok (2001). The increase in fecundity with body length and weight is consistent with the observations in other *Amphiprion* species (Beldade et al., 2012; Hattori, 2012), which makes body length and weight reliable indicators of the capacity of egg production (Bagenal, 1978). The histological results were alike to those observed in *A. ocellaris* as reported by many others (Moyer and Nakazono,1978; Madhu et al., 2010; Abol-Munafi et al., 2011). The present study showed that the ovaries consisted of oocytes of various stages and were randomly scattered in the gonad, indicating an asynchronous ovarian development organization. Such organization in general may be found in iteroparous species with multiple spawning seasons

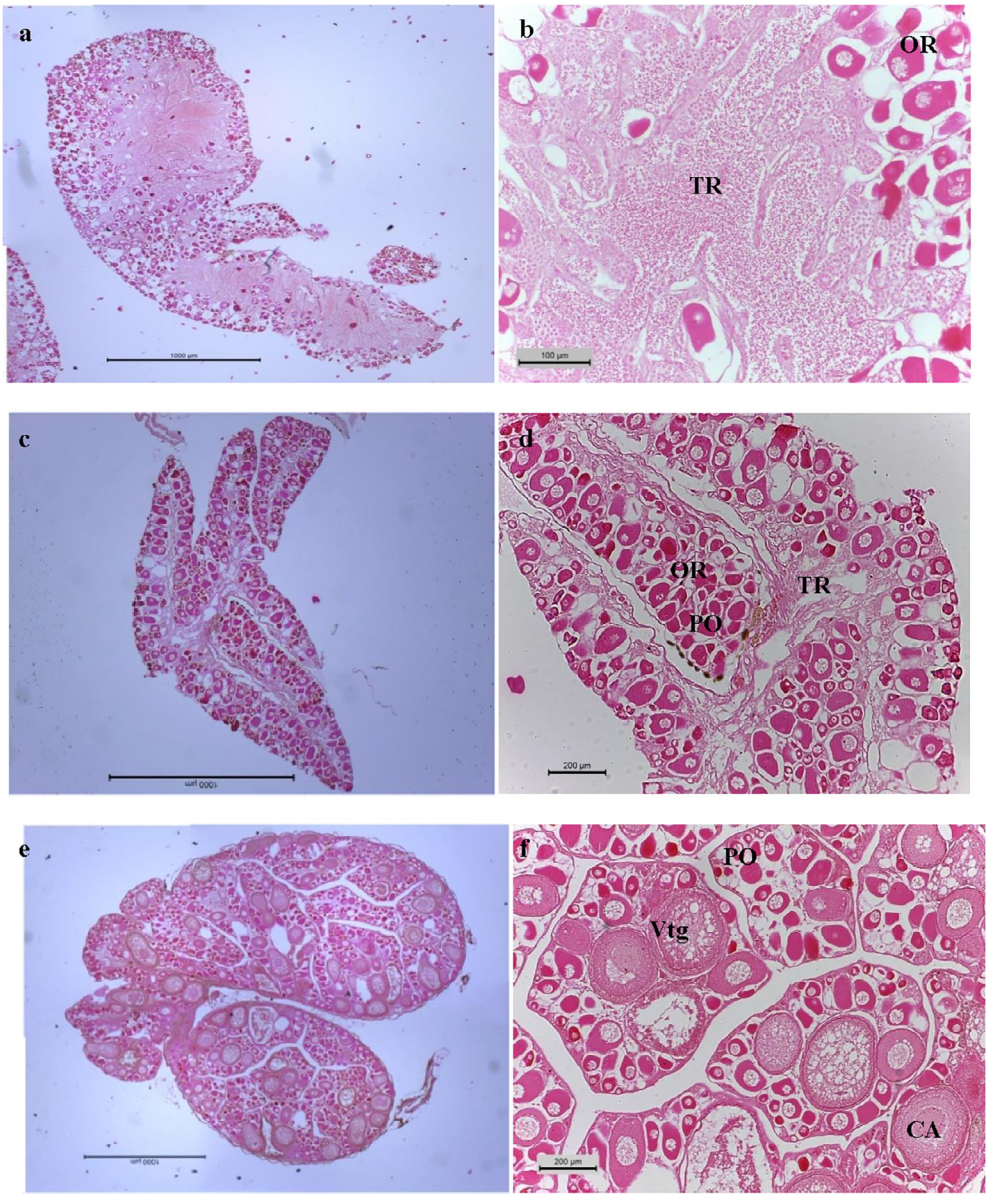

Fig. 5. Histological examination of the gonads of *A. ocellaris*. (a-b): Male ovotestes which consists of testicular region (TR) at the centre surrounded by ovarian cells (OR) at the periphery (stage I only), (c-d): ovotestes of non-breeder which consists of stage I and II oocytes (majority)(PO) and all stages of spermatogenic cells, (e-f): Mature ovary with all stages of oocytes, (PO, primary oocytes; CA, cortical alveoli; Vtg, vitellogenic oocytes).

that mostly relies on food availability in the environment at that time (Hunter and Leong, 1981), which is the case for *A. ocellaris* as they are reported to spawn throughout the year in tropical countries (Fautin and Allen, 1992).

The presence of both ovarian and testicular tissues in the gonad of male and non-breeders and the absence of testicular tissues in the ovary of the females suggested that *A. ocellaris* undergo a change of sex from male to female (protandry) that may not be reversible. Similar observations were also reported by Abol-Munafi et al. (2011) and also in gonads of other *Amphiprion* species (Moyer and Nakazono, 1978; Ochi, 1989; Miura et al., 2003; Nakamura et al. 2005; Casadevall et al., 2009). This indicates that *Amphiprion* species showed consistency in their reproduction strategy disregarding of their locality.

Our results showed that in the male ovotestes, the testicular tissues were found located at the centre while the ovarian tissues were located at the periphery. A reverse organization was observed in the male ovotestes of *A. polymnus* and *A. clarkii* where testicular tissues were peripherally located while the ovarian tissues were centrally located (Abol-Munafi et al., 2011; Ratanayuvakorm et al., 2006; Miura et al., 2003). The ovotestes in the non-breeders of *A. polymnus* and *A. clarkii* were also similar to that *A .ocellaris*, where they primarily consisted of ovarian tissues without any connective tissue between the two regions, with the exception of the ovarian and testicular regions that do not intermix as in *A. ocellaris*. The change of sex from male to female in *A. ocellaris* may not be reversible; however Madhu et al. (2010) discovered that when reared in the absence of the breeding pair, the largest non-breeder member can become female without passing through the male stage and the second largest non-breeder developed into the breeding male. Similar results were obtained by Brusle-Sicard et al. (1991) and Stahlschmidt-Allner and Reinboth (1991) in *A. frenatus*, *A. clarkii* (Miura et al., 2003; Nakamura et al., 2005) and *A. akallopisos* (Mills et al, 2018). This emphasises the importance of pairing and social structure in the sex changing mechanism of clownfish and suggests that protandry in clownfish could be facultative (Madhu et al., 2010).

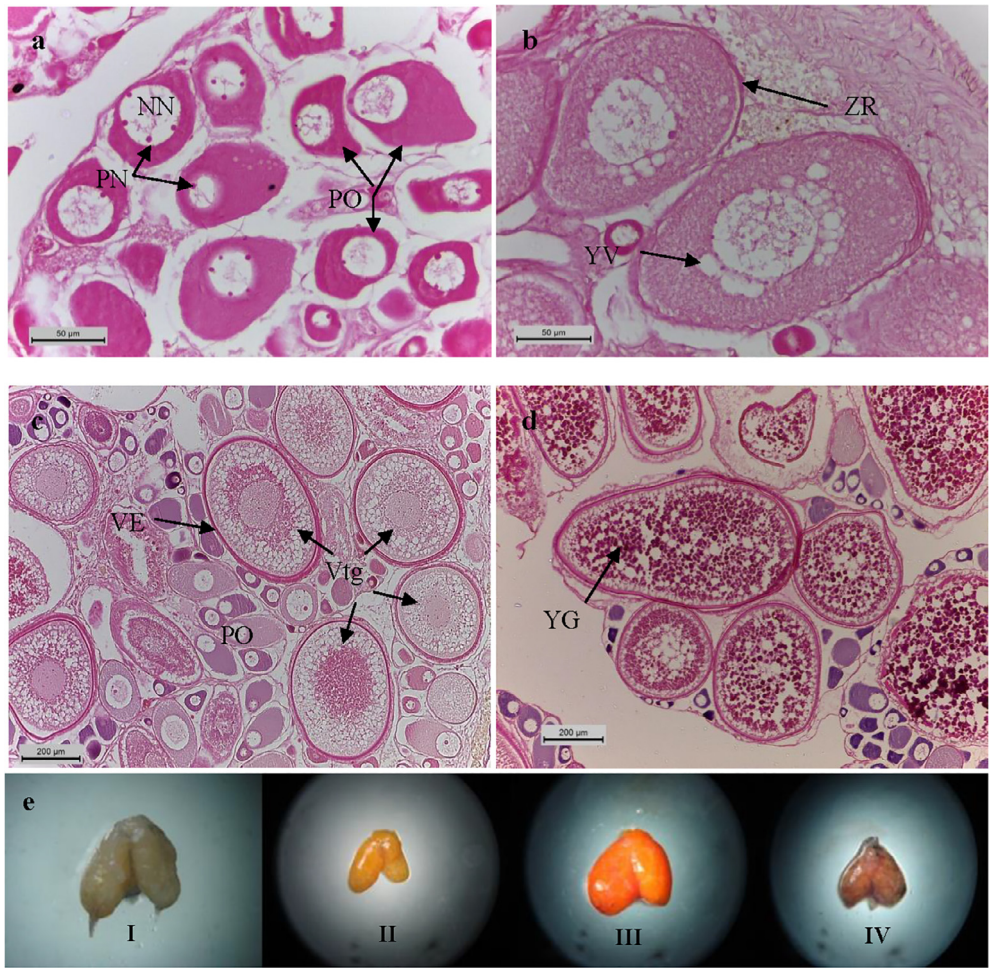

Fig. 6. Stages of oogonia; (a) stage I (Primary growth): N, nucleus; PN: peripheral nucleoli; PO, primary oocyte (b) stage II (cortical alveoli): YV, yolk vesicle; ZR, zona radiata (c) Stage III (vitellogenesis): Vtg, vitellogenic oocyte; VE, vitelline envelope; PO, primary oocyte, (d) Stage IV (oocyte maturation): YG, yolk globule and (e) The change in colours in the ovary of *A.ocellaris* in different maturity stage.

Conclusion

This study has provided a more detailed understanding of the basic diet and reproduction biology of *A. ocellaris* in Malaysian waters. *A .ocellaris* is omnivorous; stomach content consisted of variety of larva and algae. Results were further supported by trophic level analysis of 2.98 ± 0.29 with no shift in diet with increasing size. Population growth pattern for clownfish estimated from a length-weight relationship ($W = aL^b$) exhibited an isometric growth ($b = 3.0$). An estimated total fecundity of 23-1518 was obtained. Fecundity showed a positive correlation with total length, total weight, eviscerated weight and ovary weight, indicating that body size is an important indicator of the female reproductive output. Histological studies of the gonad demonstrated that the male and non-breeders have ovotestes, while females have ovaries consisting only of oocytes, suggesting protandrous hermaphroditism, although there is evidence that protandry may be facultative for *A. ocellaris*. This emphasizes the importance of social structure and pairing in regulating gonad maturation and mating success. Our findings will be useful for biologist and resources managements in formulating effective strategies for the conservation and aquaculture of *A. ocellaris* in Malaysia.

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