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Histological Study on the Gonad of the Protandrous Anemonefish (*Amphiprion ocellaris*)

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Abstract: Standard histological protocol were performed on fifteen families of *A. ocellaris* collected at Pulau Bidong, Terengganu to describe and differentiate the gonad morphology between male, female and the non-breeders of protandrous Anemonefish (*Amphiprion ocellaris*). Based on the observations, non-breeder fish (3.5-5.8 cm TL) possess intermixed ovotestes with no clear boundaries between testicular and ovarian tissues. The ovotestes contained previtellogenic oocytes interspersed with fewer spermatocytes. Male fishes (5.3-6.9 cm TL) exhibited a different type of ovotestes. Male fish ovotestes were dominated by spermatogenic cells with fewer numbers of surrounding previtellogenic oocytes. In contrast, female fish gonadal tissue (6.0-9.2 cm TL) only contained ovarian tissues with no evidence of any testicular tissues. The ovarian was also larger and more mature than male and non breeders ovarian tissue with well organised ovigerous lamellae filled with oocytes visible at different developmental stages. The absence of the testicular tissues in the female gonad shows that the change of sex from male to female is an irreversible process.

Key words: Gonad morphology, ovotestes, protandrous, anemonefish, Malaysia

INTRODUCTION

Malaysia is one of the world's largest producers of ornamental fish. Based on the 2004 Fisheries Statistics, the total production of ornamental fish in Malaysia increased 8.6% from RM 97.64 million in 2003 to RM 106.03 million in 2004 (Department of Fisheries Malaysia, 2005). However, the aquarium trade in Malaysia is almost exclusively focused on freshwater species. Malaysia has a rich and diverse array of coral fish species that have a high potential to be commercialised. Attempts at the propagation of marine fish species for ornamental purposes are severely limited due to the lack of biological knowledge and technical skills.

The anemonefish, *Amphiprion ocellaris* is one of the most popular marine ornamental species among hobbyist. This species has a high market value due to its unique body colouration and ability to breed in captivity (Newcomb and Fink, 2004). This bright orange-coloured fish with three white bars on its body inhabits coral reef areas (Allen, 1974) and sheltered lagoons up to a depth of 15 m (Newcomb and Fink, 2004). *A. ocellaris* can be found in the Indo-pacific region from the Ryukyu islands, Japan throughout Southeast Asia to Northwest Australia. In Malaysia, *A. ocellaris* is mainly found on coral reefs in the South China sea. It usually lives near or in anemones as part of a symbiotic relationship. The propagation of *A. ocellaris* in captivity has recently become an important trend due to both commercial purposes as well as for conservation. The number of wild *A. ocellaris* has decreased drastically due to the high demand from the aquarium trade and the deterioration of its natural habitat caused by human activities. According to Barton, ocean Reefs and Aquariums in Hawaii are one of the farms that already have success in breeding *A. ocellaris* in captivity for commercial purpose. In Malaysia, efforts to breed and raise *A. ocellaris* under captive conditions have previously shown to be successful (Liew, 2006). Unfortunately, production remains low because breeders have failed to develop broodstocks from F1 generation. *A. ocellaris* broodstocks are usually collected in pairs from the wild.

Problems arise in identifying the sex of the F1 fishes because *A. ocellaris* is a protandrous hermaphrodite. Sex identification is critical in *A. ocellaris* production because the fish must be paired for breeding (Liew, 2006). A protandrous hermaphrodite such as *A. ocellaris* has the ability to change sex from male to

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female. This change occurs at maturity in order to meet the requirement of their complex social structure. In nature, a group of clownfish inhabiting an anemone will consist of 4-6 fish. There will be a breeding pair (male and female) in addition to several non-breeders. The non-breeding individuals progressively decrease in size further down the social hierarchy. If the female dies or removed from the group, the male's gonads cease to function as testis and the egg-producing cells will become active. Simultaneously, the largest non-breeder will become a functioning male (Fautin and Allen, 1992).

In order to seek solutions for the problem of sex identification in *A. ocellaris* basic information, especially on the gonad structure must be gathered in order to better understand its sexual differentiation process. This study was conducted in order to describe and identify the differences in the morphology and gonad structure between female, male and non-breeders of *A. ocellaris* through histological studies.

MATERIALS AND METHODS

Species source: Fifteen *A. ocellaris* groups were collected at Pulau Bidong and Pulau Karah, Terengganu, Malaysia. Each groups consisted of 4-6 individuals.

Sample preparation: The weight and standard length was measured and recorded. Fish were humanely sacrificed using the pithing technique. The gonads are then carefully removed under a dissecting microscope before they were fixed in Bouin's solution for 24 h. Fixed samples were then preserved in 70% ethanol at room temperature prior to histological studies.

Histological studies: The prepared samples were placed in cassettes, dehydrated (tissue processing) and embedded in paraffin wax following Drury and Wallington (1980), Hinton (1990) and Kiernan (1990). Routine thin section paraffin histology was performed. Sections were 4-5 μm in thickness.

Each of the sections was dewaxed, dried and stained with hematoxylin-eosin. The slides containing the sections were then mounted before being observed under a compound microscope equipped with Motic camera and analysed with Motic Image software using a computer.

RESULTS AND DISCUSSION

The size of the non-breeder fish in this study ranged from 3.5-5.8 cm in Total Length (TL) and 0.9-3.5 g in weight. Ovotestes were found inside the body cavity in the caudal region adjacent to the intestine and below the gas bladder.

The ovotestes were flat and looked similar to the testis except for the colour and size where they were translucent and smaller than the testis. Histological examination revealed that the non-breeders possessed intermixed ovotestes with no clear boundaries between the testicular and ovarian regions with the majority of the ovotestes consisting of ovarian tissue (Fig. 1a and b). The ovarian region of the ovotestes consisted of oogonia and mostly pre-vitellogenic oocytes while spermatogonia and spermatocytes formed the testicular region (Fig. 1c). Spermatids were also found in the testicular regions of some samples (Fig. 1d). No ovarian cavity or connective tissues were found in any of the samples.

In male fishes, the size of samples from each family ranged from 5.3-6.9 cm in total length and they weight from 2.1-5.95 g. Mixed ovotestes located at the caudal portion of the body cavity were found in male fishes as were the ovotestes in the non-breeders. When compared to the ovotestes of the non-breeders the males ovotestes were larger in size. The connected ovotestes of male *A. ocellaris* were milky white in colour, flat and had an oval shape similar to the testis that can be found in many other fish species. Histological features showed that the ovotestes primarily consisted of spermatogenic cells at all developmental stages such as spermatogonia, spermatocytes, spermatids and spermatozoa (Fig. 2a). All male fish examined in this study were determined to be mature based on the presence of spermatozoa. Oogonia and pre-vitellogenic oocytes were observed in the ovarian tissue of the ovotestes (Fig. 2b).

The testicular tissue of the ovotestes was located more centrally while the ovarian tissue was peripherally located (Fig. 2c and d). Connective tissues or an ovarian cavity were not found in the ovarian and testicular regions.

Female fish examined in this study ranged from 6.0-9.2 cm in total length and weight around 5.5-13.5 g. The ovary consisted of two connected sacs filled with orange coloured eggs. Each ovary was positioned in a similar manner to the ovotestes in the non-breeders and male fishes. Initial observations showed that the ovaries of mature females (7.5-9.2 cm TL) and immature females (6.0-7.2 cm TL) were the same both in shape and colour. However, further investigations revealed that the ovary of the matured fish was much larger than the immature females. Histological examination showed that the ovary of the mature female fish contained well organised

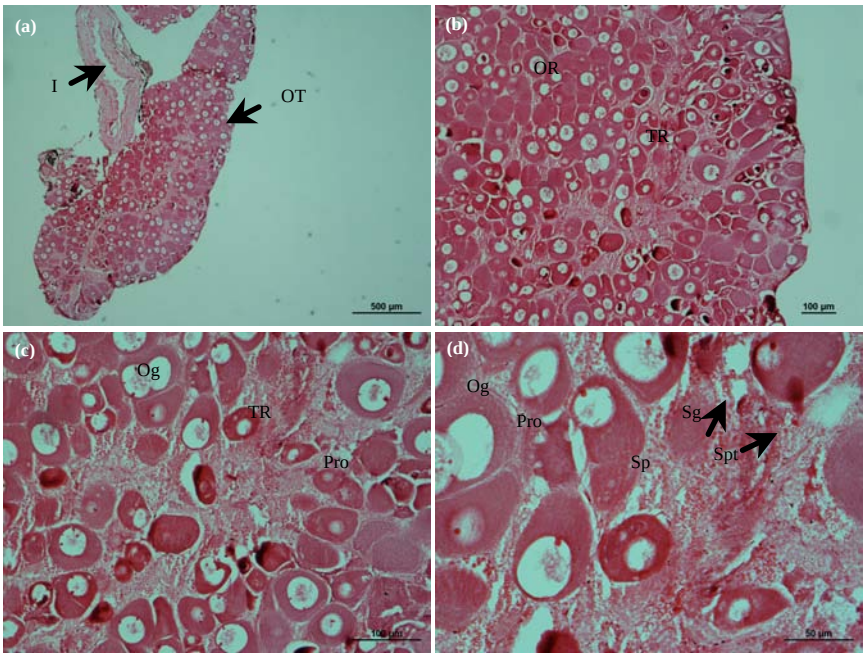

Fig. 1: Ovotestes of *A. ocellaris* non-breeders; a) non-breeders ovotestes and intestine; b) the testicular region and the ovarian region intermixed with no clear boundaries; c) a closer look at the regions and cells and d) ovotestes having spermatogonia, spermatocytes, spermatids, pre-vitellogenic oocytes and oogonia (I: Intestine; OT: Ovotestes; TR: Testicular Region; OR; Ovarian Region; OG: Oogonia; Pro: Pre-vitellogenic oocytes; Sp: Spermatocytes; Spt: Spermatids; Sg: Spermatogonia)

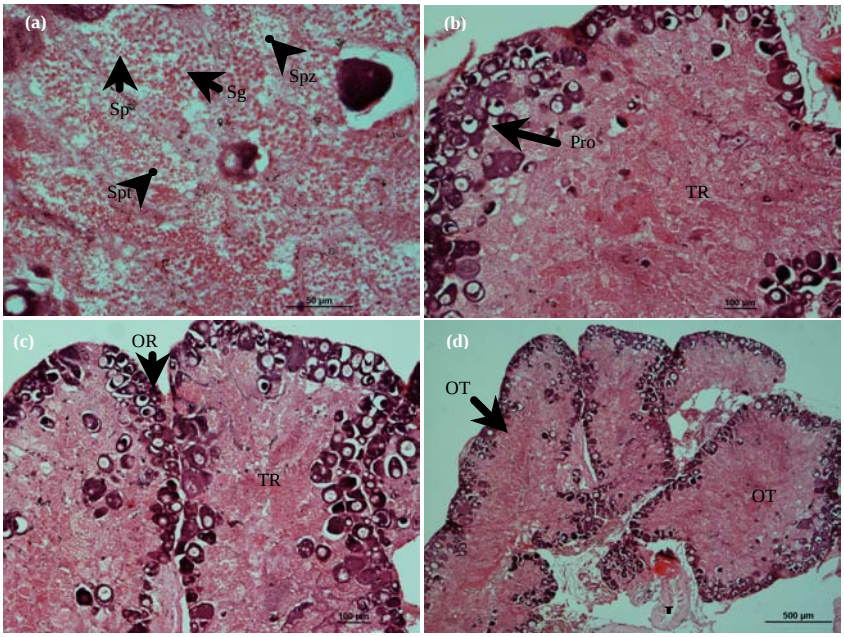

Fig. 2: Ovotestes of male *A. ocellaris*; a) ovotestes containing spermatogonia, spermatocytes, spermatids and spermatozoa; b) the testicular region surrounded by pre-vitellogenic oocytes; c) the mixed testicular region and the ovarian region and d) male ovotestes and intestine (I: Intestine; OT: Ovotestes; TR: Testicular Region; OR; Ovarian Region; Pro: Pre-vitellogenic oocytes; Sp: Spermatocytes; Spt: Spermatids; Sg: Spermatogonia; Spz: Spermatozoa)

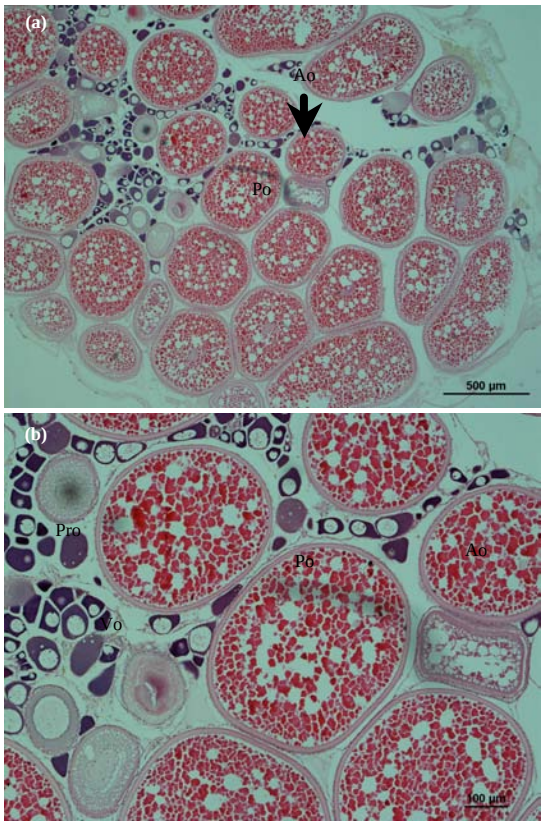

Fig. 3: Mature ovary of female *A. ocellaris*; a) post vitellogenic oocytes dominating the ovary of a mature female and b) different stages of oocytes in an ovary of a mature female (I: Intestine; OV: Ovary; Pro: Pre-vitellogenic oocytes; Vo: Vitellogenic oocytes; Po: Post vitellogenic oocytes)

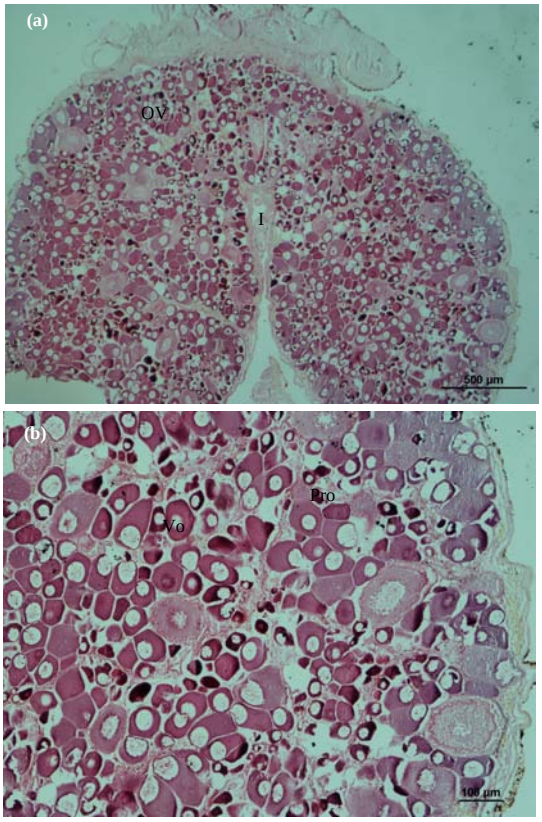

Fig. 4: Immature ovary of female *A. ocellaris*; a) ovary of an immature female and b) oocytes at different stages in the ovary of an immature female (I: Intestine; OV: Ovary; Pro: Pre-vitellogenic oocytes; Vo: Vitellogenic oocytes)

ovigerous lamellae filled with oocytes at all developmental stages (Fig. 3a). Post-vitellogenic oocytes were the most common cell, the ovary confirming the maturity of the female fish (Fig. 3b). In immature females, previtellogenic and vitellogenic oocytes were the most prevalent cells in the ovarian tissue (Fig. 4a and b). In both mature and immature female gonadal tissue, there was no evidence of any testicular tissue.

According to histological examination, the ovotestes of *A. ocellaris* non-breeders are similar to the ovotestes of the *Amphiprion polymnus* (Rattanayuvakorn *et al*., 2006), *Amphiprion clarkii* (Miura *et al*., 2003) and *Amphiprion melanopus* juveniles. The ovotestes of all this species have testicular and ovarian tissue at similar stages to the ovotestes of *A. ocellaris* non breeders. The ovotestes in both species are also primarily constructed of ovarian tissue without any connective tissue between the testicular and ovarian regions. However, the ovarian and testicular regions in both species are not intermixed as is the case for *A. ocellaris* ovotestes. The presence of developing ovarian and testicular tissues in the ovotestes of *A. ocellaris* non-breeders shows that they might have the ability to change sex directly to female without first changing to male. This ability has been observed in *A. clarkii* where several study have proven that the non-breeders of *A. clarkii* directly change into a female without turning into a male first.

Based on Davison in protandric species, the development of male cells is both preceded and followed by ovarian development where it has been argued that the default sex for fish is female. This might explain the condition of *A. ocellaris* non-breeders ovotestis where the ovarian region dominates the ovotestis. Though, the age of the samples is unknown but it is safe to assume that the non-breeders ovotestis have just started to developed. In the male fish ovotestes, the ovarian tissues are found in the peripheral regions of the gonad while the testicular tissues are more centrally located.

This configuration was reversed in male ovotestes of *A. polymnus* and *A. clarkii* where the ovarian tissues are more centrally located while the testicular tissues are peripheral. All males in the three species however have similar characteristics features of their ovotestes including an absence of connective tissue between the regions and the same developmental stage of spermatogenic cells. The prominence of testicular tissues in the male ovotestes is typical and can be found in the ovotestes of functioning males in other species of protandric fish (Hesp *et al*., 2004).

When comparing the ovotestes of the non-breeders and males of *A. ocellaris*, it is clearly evident that they are easily distinguishable. Although, the ovotestes have a similar shape, the size and colour of the two types of ovotestes differ dramatically with the non-breeders possessing small and translucent ovotestes while the male ovotestes are larger and are a milk white colour. Both ovotestes are also totally different histologically. The intermixed ovotestes of the non-breeders consisted primarily of ovarian tissues, although both sex tissues were still at the developing stage. The male mixed ovotestes, however were dominated by testicular tissues at all developmental stages while the smaller ovarian region consists of previtellogenic oocytes.

Some of the notable characteristics in the ovotestes of both non-breeders and male in *A. polymnus*, *A. clarkii* and *A. ocellaris* are different from the ovotestis of other protandric fish species. For example, the ovotestes of the blackspot seabream juveniles (*Pagellus bogareveo*) contain a cavity lined by a single layer of primordial germ cells, enveloped by a thick layer of connective tissue (Micale *et al*., 2002). The ovotestes of inactive individuals of Hawaiian sand burrowers *Crytallodaites cookei* and *Limnitchys donaldsoni* display ovarian and testicular regions that are approximately equal in the cross-sectional area. Both tissue regions are divided by a connective tissue barrier (Langston, 2004). The ovotestes of the yellowfin seabream, *Acantophagrus latus* are comprised of paired bisexual gonads consisting of a mediodorsal ovarian zone and a latero-ventral testicular zone separated by a wall of connective tissue (Abou-Seedo *et al*., 2003; Hesp *et al*., 2004).

The ovarian tissue in a female *A. ocellaris* display many similar characteristics as the female gonadal tissue in *A. polymnus*, *A. clarkia*, *A. melanopus* and the functioning females of other protandric species such as *Acanthopagrus latus* (Hesp *et al*., 2004). No spermatogenic cells were found on the ovary of all the samples. However, the lack of reproductive tissue may be caused by the degeneration of testicular tissues as describe in *A. polymnus* (Rattanayuvakorn *et al*., 2006) and *A. clarkii* (Miura *et al*., 2003). This conditions have also been mentioned by Moyer and Nakazono in their studies. The absence of testicular tissues in the ovary shows that the transition of sex from female to male is an irreversible process which is common in clownfish species. The female ovary is readily differentiated from the ovotestes of both non-breeders and functioning males due to obvious morphological differences.

CONCLUSION

The gonadal tissue of the non-breeders, male and female *A. ocellaris* are distinctly different morphologically and histologically. The gonads can be differentiated based on size, shape, colour and via histological examination. The non-breeders of *A. ocellaris* have small intermixed ovotestes without any clear boundaries between the regions with ovarian tissues dominating them. Male fish possesses mixed ovotestes dominated by testicular tissue at all developmental stages. The testicular tissue is centrally located with surrounding peripheral ovarian tissues. In contrast, female fish possess large ovaries with oocytes at all developmental stages with no testicular tissue present in both immature and mature gonad.

RECOMMENDATIONS

Further research is necessary to clarify the gonadal development in *A. ocellaris*. Studies must be conducted to evaluate the social and environmental factors as well as the role of sex steroids in the sex determination mechanism of *A. ocellaris* before the broodstock production problems can be solved.

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