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## Development of gonads and evidence of protogyny in Spangled emperor (*Lethrinus nebulosus*) from the coastal waters off Negombo, Sri Lanka.

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**Abstract** Gonad development and maturation of the spangled emperor *Lethrinus nebulosus* (Forsskål, 1775) inhabiting coastal waters off Negombo, Sri Lanka was investigated to understand the reproductive strategies. Sexual ontogeny was investigated using histological examinations of the gonads and changes in sex ratio with body size. Cellular characteristics and the most advanced germ cells presented in the gonads were considered for determining the sex and maturity stages. The transitional gonads were characterized by changes in internal gonad structure such as the appearance of spermatocyte and degenerated oocytes in the same developmental stage. Thick strands of stromal tissue within the lamellae and branched secondary strands were observed in transitional individuals. Increased blood vessels and sperm sinuses within the gonad wall were also an indications of sex transition. Therefore, *L. nebulosus* living in coastal waters off Negombo exhibited an early indication of protogynous hermaphroditism among adults. The sizes at 50% sexual maturity of males and females were estimated to be 29.5 cm and 39.5 cm respectively. A dramatic change in the sex ratio of the adult size classes suggested that female to male sex transition observed between 28.5- 40.4 cm body sizes. Sex ratios were 1.23: 0.46: 1 for female: transitional: males indicating slightly female-biased overall sex ratios. Female dominance in smaller size classes and male dominance in larger size classes were also evident Mean size at first capture recorded for *L. nebulosus* (19.6 cm) was well below the mean sizes of maturity for both male and female. Size-specific differences in sex ratios and reproductive potential indicate that conventional regulations that impose size limits for capture would not be very effective for the management of *L. nebulosus*.

**Keywords:** Spangled emperor, *Lethrinus nebulosus*, Maturation, Gonad development, Protogynous hermaphroditism, Sex transition

### INTRODUCTION

Fish may be either gonochoristic (separate male and female throughout life) or hermaphroditic (complete or partial reproductive organs of both male and female). Hermaphroditic individuals may function as males or females either simultaneously or sequentially. Sequential hermaphrodites may be protogynous in which females change to males, or protandrous in which males change to females as adults (Hoar & Randall, 1969; Jennings et al., 2005; Molloy et al., 2007). There are increasing evidences that several reef-associated fish change sex during their lifetime (e.g., Young and Martin 1982; Asoh et al. 2001; Kulmiya et al. 2002; Bean et al. 2003; Grandcourt et al. 2006; Mackie et al.

2006; McBride and Johnson 2007; Pears et al. 2007).

The family Lethrinidae (emperor fishes and large-eye breams) is one of the most common fishes exploited in artisanal demersal fisheries of the tropics (Ebisawa and Ozawa 2009). Reproductive studies have identified several lethrinid species as protogynous hermaphrodites (e.g., Thresher 1984; Sadovy 1996; Ebisawa 2006; Sadovy de Mitcheson and Liu 2008; Motlagh 2010a) resulting in female-biased adult sex ratios (Sadovy 1996).

The spangled emperor *Lethrinus nebulosus* (Forsskål 1775) is a tropical fish distributed throughout the Indo-West Pacific from the Red Sea and East Africa to southern Japan and Samoa, which can reach up to 80.0 cm total length (TL)



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and up to 8.4 kg weight (Randall 1995). It is found to inhabit coral reefs, seagrass beds, mangroves and nearshore waters up to 75 m depth. Adults are found as solitary or in small schools, whereas juveniles form large schools in shallow, sheltered sandy areas, often associated with seagrasses, algae, or sponge habitats (Randall 1995).

Previous studies on the reproductive biology of *L. nebulosus* in Northern Australia (Young and Martin 1982), Japan (Ebisawa 1990), Great Barrier Reef, Australia (Grandcourt 1999; Currey et al. 2013), Egypt (Red Sea) (Salem 1999; Mahmoud 2009), Persian Gulf and Oman Sea (McIlwain et al. 2006; Motlagh et al. 2010b), Western Australia (Marriott et al. 2010), Arabian Gulf (Grandcourt et al. 2010) have shown greater variations among studies. Young and Martin (1982) concluded that protogynous hermaphroditism is the typical mode of sexuality with no clear relationship between sex ratio and size for *L. nebulosus* found in North-Western Australia. However, a linear relationship between size and sex ratio was demonstrated for other lethriniids where females predominate at smaller sizes and males at larger sizes. There was also no clear evidence of sex change in spangled emperors within the size range 17-54 cm in the Great Barrier Reef (McPherson et al. 1988). However, other studies (e.g., Ebisawa 1990; Grandcourt 1999; Marriott et al. 2010) have shown juvenile protogynous hermaphroditism, where the transition from the ovary to testis occurs before ovarian maturation, and not the true sex-reversal often observed in protogynous hermaphrodites.

A good understanding of reproductive biology is imperative for the management of fish species mainly because of the potential disruption to reproduction caused by fishing. Stock assessment and management strategies are traditionally developed for gonochoristic species with equivalent male to female sex ratios. A better

understanding of reproductive biology is therefore important and different management trajectories are needed for those species with different reproductive characteristics (Sadovy 1996).

Issues may arise when excessive fishing is exerted on hermaphroditic populations (Bannerot et al. 1987). In protogynous species, where males tend to be larger than females, size-selective fishing may result in sperm limitation due to the differential loss of larger, older males (Koenig et al. 1996). Some simulation studies have shown a failure to account for the selectivity characteristics in relation to size at maturity and the size at sex change can lead to unexpected collapse of populations with protogynous sexual pattern (Alonzo and Mangel 2005).

There are no previous studies on the reproductive biology of *L. nebulosus* inhabiting in Sri Lankan coast of the Indian Ocean, and the present study was to augment this knowledge gap.

## MATERIALS AND METHODS

*L. nebulosus* samples were collected from artisanal fishers in two of the fish landing sites in Negombo (Fig. 1) in monthly intervals between August 2015 and February 2016. Body size (Standard Length, SL) was measured to the nearest centimetre using a measuring board for all *L. nebulosus* found in the landing sites during the sampling day, and body weight was recorded to the nearest gram with an electronic scale. All together 430 specimens were sampled out of which 70 specimens representing different size classes were used for histological preparations of gonads.



**Fig 1** Fish landing sites where the samples were obtained and the fishing area.

Since it was not possible to identify the gonad maturity stages macroscopically, histological analyses were carried out. In the laboratory, gonads were dissected, and weighed to the nearest gram and preserved in FAACC (Formaldehyde 4%, Acetic Acid 5%, Calcium Chloride 1.3%) solution following Mackie (2006). Series of transverse thin medial sections of 5-7 $\mu$ m were obtained from each gonad using a rotary microtome. Sections were mounted on glass slides, stained with Heidenhain's iron hematoxylin and eosin and permanent mounts were prepared using standard histological techniques. Preparations were examined microscopically using high power ( $\times 100$ – $\times 400$ ) with a Microscope Image Projection System (MIPS, Magnus<sup>®</sup> Analytics).

Ovaries were assigned to sexual development stages based on the most developed oocytes as well as the presence or nature of intra-lamellar stromal tissue, the relative thickness of the ovary walls and muscle bundles, atretic oocytes, postovulatory follicles and yellow-brown bodies (Adams et al. 2000; Marino et al. 2001; Bean et al. 2003; McBride and Johnson 2007). The testes were classified into maturity stages based on the classification of Grier and Taylor (1981). The transitional individuals were confirmed when the gonad contains both degenerating mature female tissue and proliferating male tissue (Bean et al. 2003).

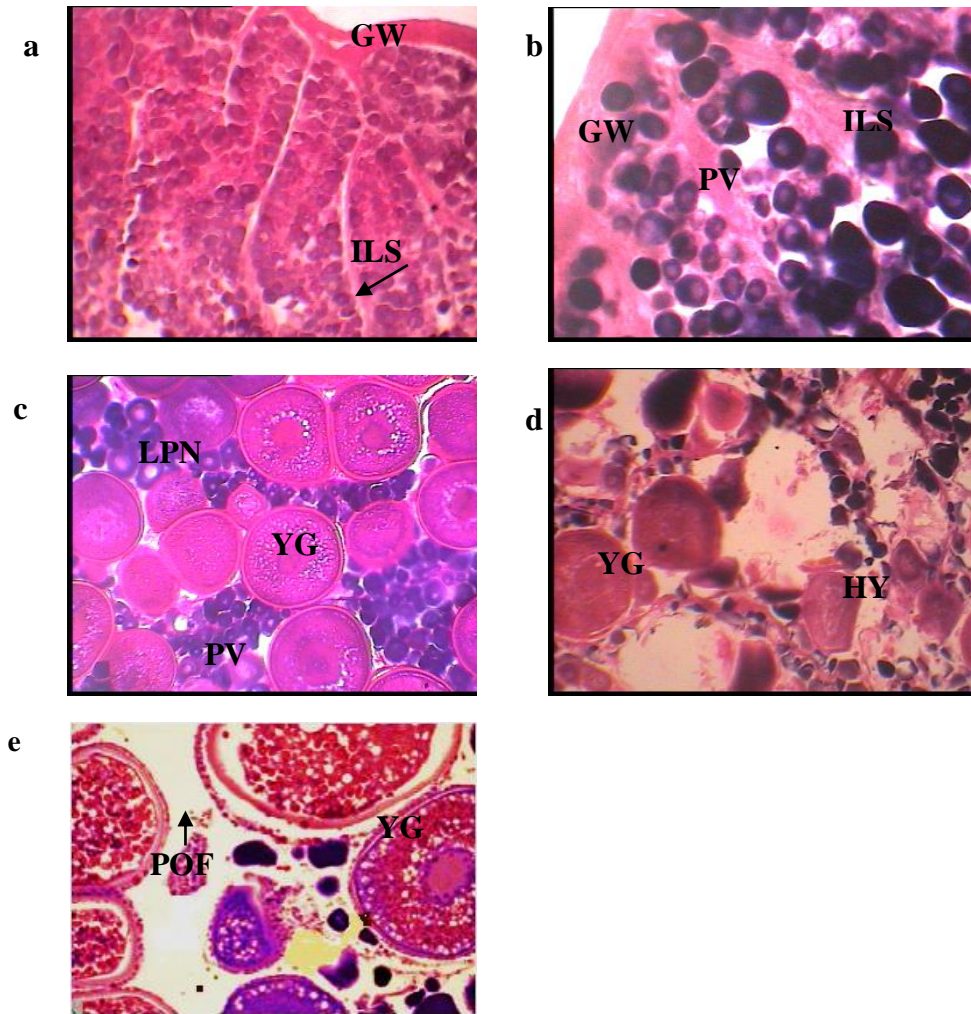
The mean length at first sexual maturity ( $L_{m50}$ ) for both males and females was estimated by considering the proportion of mature fish in each 4 cm size-class. The sex ratio was calculated for all males and females separately and also for 4 cm size intervals. Frequency plots were used to

demonstrate the distribution of each sex over the size ranges.

## RESULTS

### Ovary structure

Macroscopic investigations showed that *L. nebulosus* ovaries were cylindrical with approximately equal size. Microscopic investigations revealed five developmental stages as immature, resting, ripe, running ripe, and post-spawning. Immature ovaries were dominated by pre-vitellogenic oocytes, and strands of stromal tissue within the lamellae were filamentous (Fig. 2a). Resting ovaries were dominated by pre-vitellogenic oocytes. Primary growth oocytes of resting ovaries had a thick wall. Strands of stromal tissue within the lamellae were usually thin and filamentous, although in some cases, these tissues showed signs of thickening and were associated with an increased number of intra-lamellar blood vessels (Fig. 2b). Ripe ovaries were in a state of active vitellogenesis with yolk globule, and oocytes belong to all stages of development were present. Strands of stromal tissue within the lamellae were thin and filamentous (Fig. 2c). Running ripe ovaries contained hydrated oocytes, yolk globule, and migratory nucleus stage oocytes were also present (Fig. 2d). In postspawning ovaries, yolk globule oocytes were present in the lamellae with postovulatory follicle (POF) often with atretic oocytes (Fig. 2e).

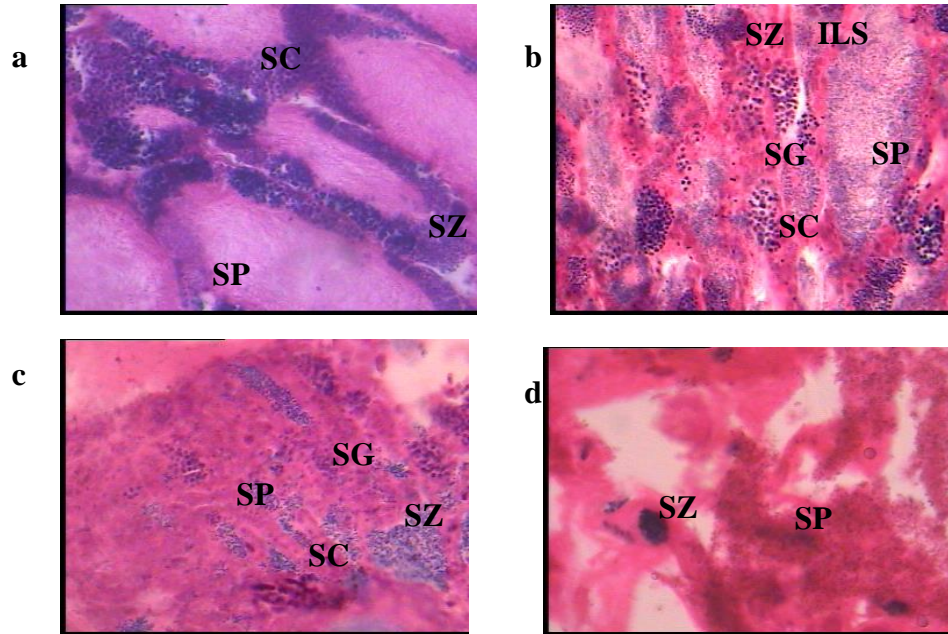


**Fig 2** Structure and cellular characteristics of developmental stages of *L. nebulosus* ovaries: (a) Immature, (x100) (b) resting, (x400) (c) ripe (x100) (d) running ripe (x100). (e) Postspawning (x200). GW, gonad wall; ILS, inter lamellar stromal strands; LPN, late peri nucleolus; PV, previtellogenic; YG, yolk globule; HY, hydrated oocyte; POF, postovulatory follicle.

### Testis structure

Four maturity stages were recorded for *L. nebulosus* testes as pre-spawning resting, ripe, spent and post-spawning resting. Pre-spawning resting testes composed of densely packed spermatocysts containing spermatogonia, spermatocytes, and spermatids. Spermatozoa occurred more frequently in spermatocysts (Fig. 3a). Ripe testes were dominated by spermatozoa

filling all sperm sinuses. Spermatocysts containing early stages of sperm development were rare (Fig. 3b). Spent testes characterized by loosely arranged tissues which were highly vascularized and containing considerably a fewer spermatocysts of any sperm stage (Fig. 3c). Post-spawning resting testes were considerably rare, and the spermatocysts were no longer loose or diffusely arranged (Fig. 3d).



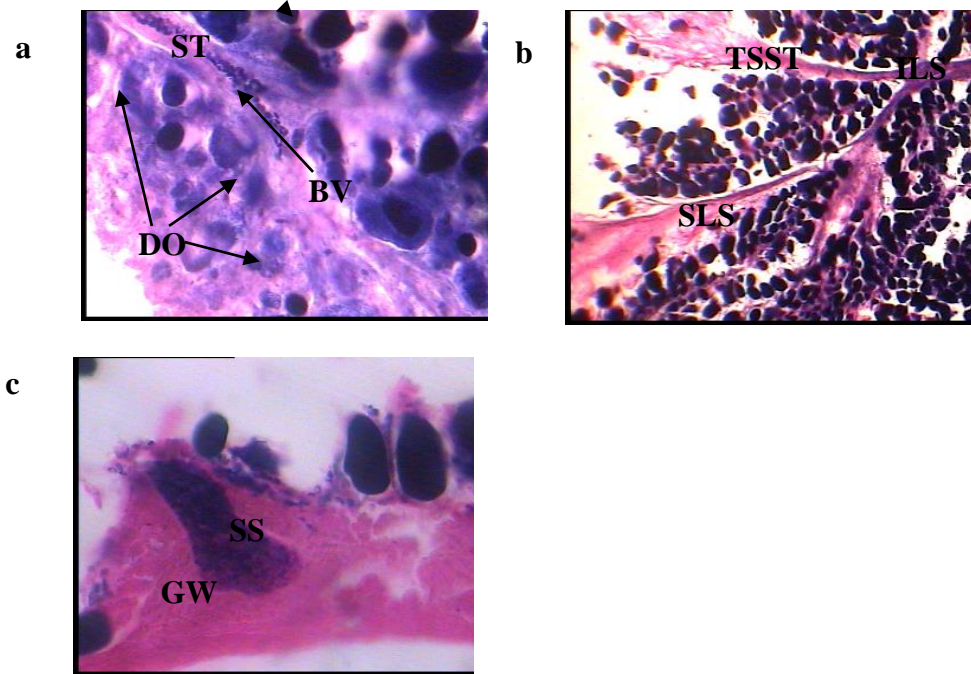
**Fig 3** Structure and cellular characteristics of developmental stages of *L. nebulosus* testes: (x400) (a) **pre-resting**, (b) **ripe**, (c) **spent** and (d) **post resting**. SG, spermatogonia; SC, spermatocytes; SP, spermatids; SZ, spermatozoa; ILS, intra lamellar stromal strands

### Transitional gonad structure

In all transitional gonads, oocytes had degenerated considerably prior to the proliferation of spermatogenic tissue (Fig. 4a). This characteristic is the first indication of sex change in many cases. In females, stromal strands appear as filamentous structures between the lamellae folds, but during the transition phase, these strands become thickened and secondary strands branch off from the main strands of stromal tissue into the surrounding tissue (Fig. 4b). These stromal strands

were well developed and highly infiltrated with blood vessels (Fig. 4a). At the distal end of the strands of stromal tissue, sperm sinuses had formed within the gonad wall (Fig. 4c).

Proliferating spermatocysts were commonly located in the peripheral margins of the lamellae. During the study, twelve specimens of *L. nebulosus* had both female tissue and spermatocysts belong to one sperm developmental stage.



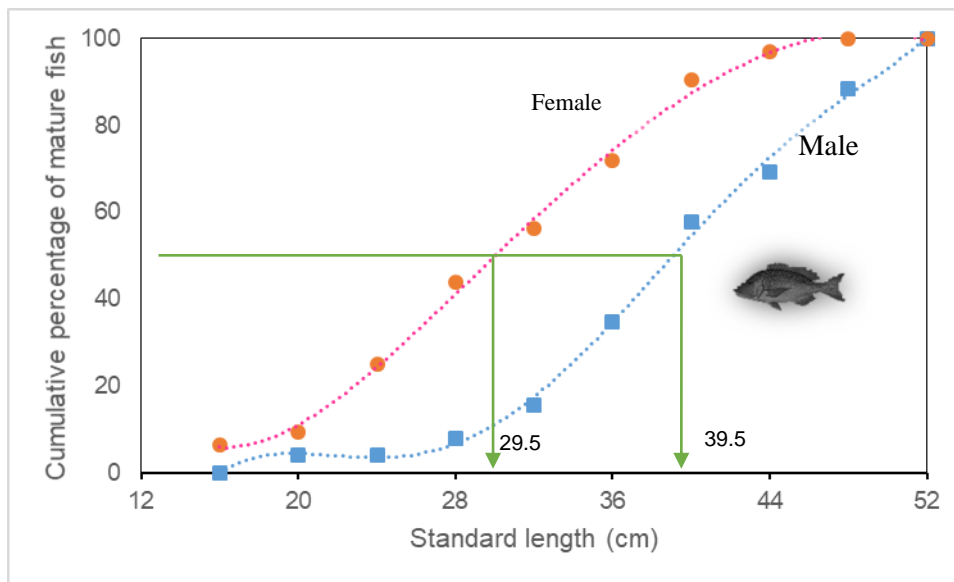
**Fig 4** Structure and cellular characteristics of transitional *L. nebulosus* gonads: (x400).

(a) Degenerating oocytes (DO) and stromal strands (ST) with increased blood vessels (BV), (b) Presence of thickened strands of stromal tissue (TSST) within the lamellae, branched secondary strands, intra-lamellar stromal strands (ILS), and secondary lamellar stromal strands (SLS), further prove that sex transition has been initiated. SS, sperm sinus; GW, gonad wall.

#### Size at maturity

According to Fig. 5, mean sizes that 50% of *L. nebulosus* males and females attained the first

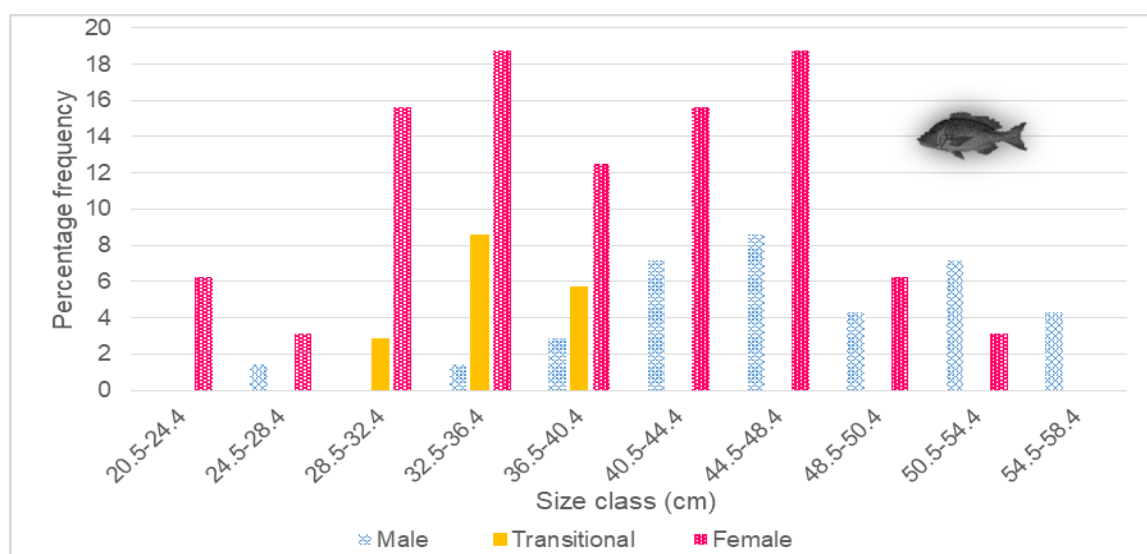
sexual maturity ( $L_{m50}$ ) were at 29.5 cm and 39.5 cm standard lengths, respectively. This indicates that males mature at smaller sizes compared to females.



**Fig 5** Cumulative percentage of mature *L. nebulosus* males and females with the size showing the mean, standard lengths at which 50% of the individuals attain sexual maturity ( $L_{m50}$ ) (N=70).

As shown in Fig. 6, sex transition occur between 28.5- 40.4 cm size classes. At this size, female *L. nebulosus* should be already or nearly matured. It is evident that the females were predominated in the smaller size classes, and they either changed sex to male or remained female and matured at a larger size whereas males were dominant in the larger size classes. The sex ratio for female: transitional: the male was 1.23: 0.46: 1, indicating slightly female biased overall sex-ratio.

When examining length frequency distribution of monthly landings, the size of *L. nebulosus* at first capture was found to be in the size class 16.5-20.5 cm (Fig. 7). The mean size at first capture recorded for *L. nebulosus*, which is 19.6 cm was well below the sizes for both male and female maturity sizes 29.5 cm and 39.5 cm respectively.



**Fig 6** Sex specific size frequency distribution (N=70).



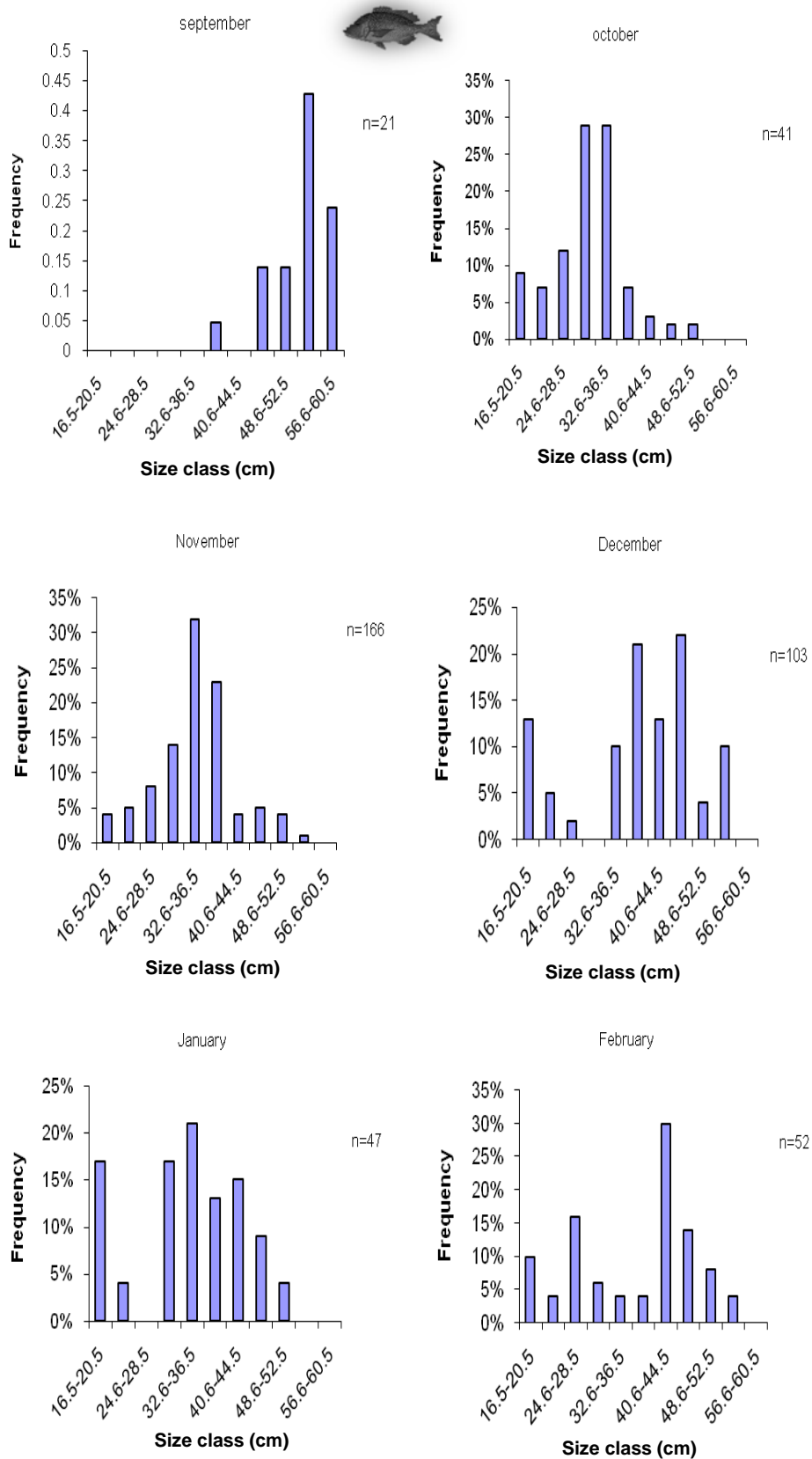


Fig 7 Monthly variation in the length frequency distribution of *L. nebulosus* landings

## DISCUSSION

All stages of mature male and female gonads studied had no specific features to identify them macroscopically. Therefore, confirmation of the sexual pattern required histological analysis of gonad morphology as with other studies (Pears et al. 2007). Five developmental stages were identified for ovaries and four stages for male *L. nebulosus* in this study. Oocyte and sperm developmental stages of *L. nebulosus* was in-line with few other similar studies (Adams et al. 2000; Asoh et al. 2001; Marino et al. 2001; Bean et al. 2003; Knight et al. 2007).

Sex change, where individuals function as both sexes sequentially, is a common reproductive strategy in tropical reef fishes, occurring in nearly 50% of families (Jennings et al. 2005; McIlwain et al. 2006; Molloy et al. 2007). The simultaneous presence of developing sperm crypts and degenerating mature female tissues and peripheral sperm sinuses described here suggest that sex change in *L. nebulosus* is consistent with patterns described for other protogynous hermaphroditic reef fishes (Adams et al. 2000; Kulmiye et al. 2002; Bean et al. 2003). According to Adams et al. (2000), the presence of other features such as strands of stromal tissue within the lamellae, increased number of blood vessels along the stromal strands and within degenerating female tissue during sex change, are common in many protogynous reef fishes. During the study, 12 specimens of transitional stage *L. nebulosus* were identified among the sample subjected to histological examination. The presence of strands of stromal tissue within the lamellae in *L. nebulosus* is also an important structural characteristic that appears to vary in function depending on the gonad stage. In females, stromal strands appear as filamentous structures between lamellar folds, but during the transitional phase, these strands become thickened and secondary strands branched off from the main strands of stromal tissue into the surrounding tissue and increased number of blood cells. Bean et al. (2003) described that these blood cells are primarily phagocytic and also function as nutrient cells, whose role may be both to break down existing female tissue and to provide nutrients to cells involved in male tissue development. The increased number of blood vessels during sex

change may, therefore, facilitate the complete transition from female to male.

The presence of both female tissue and spermatocytes in a single developmental stage indicates the first sign of sex change in females. However, as pointed out by Bean et al. (2003), proliferating spermatocytes of more than one cell stage would be required to confirm any sex change. This study shows an early indication that sex change has initiated in *L. nebulosus*. However, further studies may facilitate the complete understanding of protogynous hermaphroditism in *L. nebulosus* inhabiting in coastal waters of Sri Lanka.

Young and Martin (1982), found evidence for protogynous hermaphroditism in eight lethrinid species from Australian waters and suggested that the protogynous hermaphroditism is a usual mode of sexuality in lethrinid fishes. However, the mechanism of controlling sex change is not clearly understood in any of the lethrinids studied. Ebisawa (1990) suggested that *L. nebulosus* was a juvenile hermaphrodite with sex being determined around 24 - 30 cm fork lengths but the fish was not maturing until approximately 40 cm fork length.

According to the present study, the mean sizes that 50 % of *L. nebulosus* males and females attain the first maturity were 29.5 cm and 39.5 cm standard length respectively, indicating that males mature at smaller sizes than females. Several other studies have shown that the *L. nebulosus* males attain sexual maturity at relatively smaller sizes. Grandcourt et al. (2010), who studied *L. nebulosus* in Arab Emirates, Abu Dhabi, found that males mature at 28.6 cm whereas females at 31.3 cm. Mean sizes of maturity of 27.6 cm for males and 28.6 cm for females were reported in the Southern Arabian Gulf (Grandcourt et al. 2006), and Mahmoud (2009) reported from the Red Sea in Egypt that mean size of maturity was 25.1 cm for males and 26.2 cm for females.

Grandcourt et al. (2006) found that males tend to be larger than females for protogynous species. The present study also revealed that the majority in the larger size classes were males, although there was considerable overlap in the size distribution of the sexes. Sex-specific size frequency distribution in the present study (Fig. 6) indicates that transition occurs within the size range 28.5- 40.4 cm when most of the females are not matured. According to Young and Martin (1982), the sexual

transformation of lethinids occurs over a wide range of sizes with female predominating in the larger size classes. Similarly, Kulmiye et al. (2002) and Jennings et al. (2005) reported female predominance in the larger size classes from New Caledonian waters. The first sex is always more abundant in fishes that change sex, thus giving female biased sex ratio for protogynous species. The present study suggested that the males were predominating in larger size classes. Also, the presence of larger females in the population demonstrates that only some females change sex while others remain as females throughout their lifetime.

Ebisawa (1990) identified intersexual gonads in juveniles of *L. nebulosus* from Okinawa waters and concluded juvenile protogynous hermaphroditism. Grandcourt (1999) also found evidence of juvenile protogyny from the Great Barrier Reef. The juvenile hermaphroditism described by Ebisawa (1990) and Grandcourt (1999) is regarded as a non-functional sex change (also referred to as functional gonochorism), which will not be followed by post-maturational sex change (Sadovy and Liu 2008). Marriott et al. (2010) from Ningaloo Marine Park, Australia demonstrated a non-functional protogynous hermaphroditic strategy. A study by Currey et al. (2013) also indicated non-functional protogynous hermaphroditism for *L. nebulosus* from Great Barrier Reef, Australia.

However, a study from the southern Arabian Gulf showed no histological evidence of adult sex change, and sex ratios did not differ significantly across size classes (Grandcourt et al. 2010). A study off the Sinai Peninsula in the Red Sea (Salem (1999) and a study in Egypt from the Red Sea (Mahmoud 2009) also revealed no evidence of any sex change from the study samples.

According to the size-frequency distribution for *L. nebulosus* landings in this study, it was evident (Fig. 7) that the smallest size of the individuals caught (19.6 cm) was immature. However, larger fish (mature) were more abundant in most of the months studied throughout the study period except for December and January. From this study it is evident that larger individuals caught in the fishery are more likely to be males. Since fishing generally targets larger fish, disproportionate removal of males would affect reproductive traits of the population, which may lead towards more female

biased populations resulting in sperm limitations. McIlwain et al. (2006) also indicated that the presence of sequential hermaphrodites would considerably complicate the management of a fishery. Therefore, studies of this nature may benefit the proper management of the already distressed fish populations. This also illustrates the importance of understanding species-specific life histories to infer responses to exploitation.

## CONCLUSION

*L. nebulosus* living in coastal waters off Negombo, Sri Lanka provides an early indication of protogynous hermaphroditism. Fifty percent of males and females attain sexual maturity between 29.5 cm and 39.5 cm standard lengths, respectively. The mean size at first capture for *L. nebulosus* landings was 19.6 cm which was well below the male and female size-at-maturity. Sex transition initiated between 28.5- 40.4 cm size classes. Female dominate in smaller size classes, and male dominance in larger size classes was evident. Overall sex-ratio was slightly female biased.

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