



Early sex change of barramundi *Lates calcarifer* farmed in Singapore

Celestine Terence^{a,*}, Jose A. Domingos^{a,b}, Joseph Angelo Uichanco^a, Yann Monteil^c,
Loïc Monteil^c, Emmanuel De Braux^c, Quyen Q.T. Banh^d, Susan Gibson-Kueh^a, Dean R. Jerry^{a,b}

^a Tropical Futures Institute, James Cook University Singapore, 149 Sims Drive, 387380, Singapore

^b Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville, 4811, Queensland, Australia

^c Barramundi Asia Pte Ltd, 35 Fishery Port Road, 619742, Singapore

^d Institute of Aquaculture, Nha Trang University, 2 Nguyen Dinh Chieu, Nha Trang, Vietnam

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ABSTRACT

Barramundi, *Lates calcarifer*, are protandrous hermaphrodites where individuals first sexually mature as male at 3–5 years of age, before reorganizing gonadal tissue to change to female several years later when they reach around 70 cm in total length (~4–5 kg). However, recent anecdotal evidence suggests that in populations farmed in Singapore, this sex change may be occurring much earlier than previously recorded, thus posing challenges for hatcheries to select and maintain male fish for breeding purposes. This study examined the gonadal sex and maturation status of barramundi from a commercial sea-cage farm located in the southern waters of Singapore and confirmed this precocious sex change phenomenon. Barramundi (n = 173) harvested between 2 and 3 years old were individually measured (mean ± SE); total length (70.3 ± 7.3 cm), weighed (4.7 ± 1.4 kg), and had their gonads dissected for sex identification through histological analyses. Histology showed that only 19% were still male, 8% were in transitional stages of sex change and 73% of fish were female. In this precocious farmed population, females as small as 2 kg were found, with the proportion of males in the population decreasing to less than 50% from 2.5 to 3 kg (55–60 cm). Above 3 kg and 5 kg, 70% and 90% of sampled individuals were females, respectively. Comparative farm studies from French Polynesia and in wild Australian stocks indicate that the farmed population in our study is changing sex at body weights approximately half that of what was previously known. The findings of this study are important for local breeders to make decisions of when, and at what size to select candidate *L. calcarifer* broodstock in Singaporean waters so that the recommended 2:1 male-to-female spawning ratio can be achieved. The exact causes for this early sex change are still unknown and deserve further investigation.

1. Introduction

Barramundi, or Asian seabass (*Lates calcarifer*), is an important tropical food fish species that is widely farmed in Southeast Asia, Australia, the Middle East, and increasingly in the United States of America (Jerry, 2013). Barramundi are protandrous hermaphrodites, first maturing as male at the age of 3–5 years old, before changing sex into females (Davis, 1982), although earlier fisheries data suggested for the potential rare presence of primary females (Moore, 1979). The initial testicular differentiation of the species starts around 44 days-post-hatch (dph) and proceeds until around 140 dph (from 25.8 ± 3.3 mm to 169.4 ± 40.3 mm in total length, respectively) (Banh et al., 2017) confirming that the species primarily first matures as male. Such a sequential hermaphroditic characteristic adds complexity to the captive

breeding when compared to fish that are gonochoristic and thus do not change sex. For this reason, younger males are selected as broodstock candidates before they transition to females and are put together with older and larger females, most often from a previous generation (Robinson and Jerry, 2009).

Barramundi hatcheries generally employ a 2 male : 1 female sex ratio in broodstock tanks to ensure effective fertilization (Bensam and Nammalwar, 1991). However, this sex ratio eventually becomes female-biased as the males in the population change sex, affecting breeding performance and fry output (Schipp et al., 2007). Recent efforts to better understand the molecular mechanisms of sex differentiation and sex change in barramundi have found that several genes which have important reproductive functions (e.g. sex determination, steroidogenesis, NF-κB related functions, germ cell markers, production of

* Corresponding author.

E-mail address: celestine.terence@jcu.edu.au (C. Terence).

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retinoic acid, and *Wnt* signaling pathways) in mammals and other vertebrates, have a sexually dimorphic expression pattern in the testes and ovaries. Pathways that are involved in the well-known zebrafish ovary-to-testis transformation are the same as those involved in the barramundi testis-to-ovary transformation, despite reversal of direction (Ravi et al., 2014). Moreover, two of the well-studied sex-related genes, the male-biased Doublesex and Mab-3 related transcription factor 1 (*dmrt1*) and the female-biased aromatase (*Cyp19a1a*) were found to play a major role in this process given that they are differentially methylated and alternatively spliced in barramundi testes and ovaries (Domingos et al., 2018). In practice, controlling sex differentiation and maintenance of the broodstock are of great importance for stable production of fry (Budd et al., 2015), and efforts to manipulate sex differentiation in barramundi through exogenous steroids (e.g. 17 β -estradiol and 17 α -ethinylestradiol) and non-steroidal factors (e.g. fadrozole) have shown great promise (Banh et al., 2017, 2021a, 2021b). It is not yet known what factors trigger the initiation of the sex change process in barramundi. However, there is some evidence that water temperature may play a role in accelerating sex change, as the species tends to initiate transition to the female gonadal state earlier than usual when exposed to higher water temperatures. Athauda et al. (2011) and Athauda and Anderson (2014) studied the effect of temperature on the sex inversion of the barramundi by culturing them in varying water temperatures ranging from 22 °C to 34 °C. They discovered that the highest gonadal aromatase and sex hormone Estradiol 17- β (E_2) activity was found in the warmer temperatures of 31 °C and 34 °C and decreased in cooler water temperatures of 22 °C–28 °C. An increasing trend in the number of transitional gonads was found as the holding temperature increased. The positive relationship displayed between temperature and reproductive hormone activity indicate that increasing culture temperature results in early transition of barramundi into females (Athauda et al., 2011; Athauda and Anderson, 2014).

Farming of barramundi is particularly important in Singapore, where the species is cultured in both marine sea cages and Recirculating Aquaculture Systems (RAS). Singaporean marine waters offer ideal conditions for farming of barramundi, with water temperatures ranging between 27–31 °C, salinity ranging from 26 to 31 ppt, dissolved oxygen levels remain above 5 mg/L, and the chemical oxygen demand (COD) does not exceed 3 mg/l (Chou and Lee, 1997). There are currently 121 fish farms in Singapore, consisting of 109 licensed sea-based leases and 12 land-based leases (Singapore Food Agency, 2019). Coastal fish farms span the West Johor Straits (off Lim Chu Kang and Kranji), Southern waters (Semakau Island) and East Johor Straits (Off Changi, Seletar, Pasir Ris, Ubin Island and Tekong Island) (Sein et al., 2020), of which a great number farm barramundi. Barramundi fry are usually obtained from Australian, regional Southeast Asian, and Singaporean hatcheries. As local production increases, commercial barramundi broodstock and hatchery facilities are now being established to meet the demand for fry and juveniles.

Recently, there have been anecdotal reports from barramundi hatchery operators in Singapore that farmed fish may be changing sex precociously, where females below 50 cm in size were observed, posing a challenge to select and condition males from sea-cages for use as broodstock. This is because broodstock populations quickly become skewed towards having a higher proportion of females, which negatively influences the success and genetic diversity of the spawn. Additionally, as barramundi males and females do not exhibit sexual dimorphism, identification of fish of a particular sex can be difficult. If demonstrated, the occurrence of high numbers of precocious females in Singaporean farmed barramundi would be unusual for the species, based on observations of sex ratios from wild and farmed barramundi in other regions (Moore et al., 1979; Davis, 1982, 1984; Guiguen et al., 1994; Roberts et al., 2021), although Davis et al., (1984) reported of a precocious population of barramundi from the Gulf of Carpentaria, Australia, where females were observed at sizes smaller than 45 cm and no males were observed at sizes above 50 cm due to stunted growth. In a wild

population from northern Western Australia, Roberts et al. (2021) recently observed that faster growing individuals change sex at younger age than those exhibiting slower growth; however, with no apparent decrease in size at sex change.

Given the importance for barramundi aquaculture in Singapore, there is a need to establish baseline information on sexual maturation, gonadal development and sex change of fish reared under local farming conditions to inform local hatcheries and selective breeding programs. In fact, there are no studies on the size-at-sex-change of farmed barramundi based in South East Asia, despite the region being responsible for the majority of global production of the species. Understanding when barramundi are maturing and then changing sex will help hatchery managers predict the size they should be selecting fish from sea cages as brooders, as well as help identify the driving factors of precocious sex-change in the species.

2. Materials and methods

2.1. Fish origin and sample location

Barramundi examined in this study ($n = 173$) were harvested between 2–3 years of age (25–32 months-post-hatch, m_{ph}) from a commercial marine sea-cage farm situated in the southern waters of Singapore. The fish stock originated from both Singaporean and Australian hatcheries and were cultured on the farm as mixed batches. Fish were sampled at one day post-harvest from an onshore seafood processing facility over a period of five months, from late September 2019 to late February 2020. Sampling comprised of measuring the following phenotypic traits *in situ*: total body weight (W), gonadal weight (GW), and gonad length (GL). Photos were taken of each fish in lateral and ventral views next to a ruler for *in-silico* image measurements of total length (L_t), body depth (BD), and body width (BW) using ImageJ software (Schneider et al., 2012). In addition, gonadal lobes were dissected and photographed; fish were sexed from the macroscopic observation of the gonads. The gonadosomatic index (GSI), Fulton's condition factor (K) and body shape index (H) were calculated as $GSI = (GW/W) \times 100$, $K = (100 \times W (g)) / L_t^3$, and $H = (10 \times BD) / L_t$, respectively.

2.2. Histology

A 2 cm³ subsample of the anterior portion of each gonad was fixed in 10 % neutral buffered formalin (NBF) for routine histological processing. Hematoxylin and eosin (H&E) stained, 5 μ m thick gonadal sections were analysed using an Olympus BX53 microscope and photographed with a DP74 digital image capture system (Olympus Corp). Gonads were classified into different developmental stages according to Guiguen et al. (1994) as testis (M1–M4), transitional (T1–T4) and ovary (F1–F4). Five apparent synchronous hermaphrodites (Davis, 1982), i.e. found having both well-developed ovarian and testicular tissues were classified as T2 (Table 1)

2.3. Statistical analysis

Body measurements among males, females and transitional fish were compared using one-way ANOVA and Tukey HSD tests ($P = 0.05$). In addition, in order to determine the body size and mass in which 50 % of the population had initiated or completed the sex change process, logit functions were used based on observed data for total length (Logit function = $-9.8 + 0.1666 \times L_t$) and weight (Logit = $-9.8 + 0.1666 \times W$), where the probability (%) at given length or weight was calculated as $1/(1 + e^{-\text{Logit}})$. Statistics were computed in SPSS version 25 (IBM).

3. Results

Out of the 200 fish sampled, a total of 173 samples (86.5 %) could be

Table 1
Gonadal maturation stages in barramundi as classified by Guiguen et al. (1994).

Sex Type	Index	Histological criterion (gonadal maturation stages)	Additional observations in this study
Male	M1	Mostly gonia (testis gonia)	
	M2	Mostly spermatocytes and spermatids (spermatogenesis)	Layers of connective tissue are also observed
	M3	Mostly spermatozoa (spermiation)	Spermatids and spermatocytes are still widely observed in testicular lobules
	M4	Testicular lobules devoid of spermatozoa (post-spawning)	
Transitional	T1	Degeneration of male testicular tissue	
	T2	Appearance of ovarian tissue with still degenerating testicular tissue	Some fish have gonads containing both vitellogenic, previtellogenic and meiotic oocytes while testicular structures containing spermatozoa and spermatids are still intact, resembling intersex fish.
	T3	Ovarian tissue <50 % within histological cross-section (no testicular tissue)	
	T4	Ovarian tissue >50 % within histological cross-section (no testicular tissue)	
Female	F1	Gonia and previtellogenic oocytes (previtellogenesis)	Layers of connective tissue are also observed
	F2	Vitellogenic oocytes <50 % within histological cross-section (early vitellogenesis)	
	F3	Vitellogenic oocytes >50 % within histological cross-section (vitellogenesis)	Some atretic oocytes are also observed.
	F4	Oocytes atresia (atretic)	

histologically classified according to their gonadal stage. Harvested fish (4.72 ± 1.40 kg; 70.4 ± 7.2 cm) that were able to have their sex verified were dominated by females (73 %), followed by males (19 %), and those in transitional stages of gonadal differentiation (8%) (Table 2). As expected for a protandrous hermaphroditic species, in general, females exhibited greater body lengths and GSI values compared to males and transitional fish. F3 females that were observed to be undergoing folliculogenesis, possessed gonads with the highest GSI (Table 2). Upon staging of the barramundi gonads, active folliculogenesis was observed in 43 % of fish classified as female (F3), while active spermiation was observed in 48 % of those classified as male (M3) (Fig. 1).

Results indicated strong evidence for precocious sexual change in Singaporean farmed barramundi. At body weights below 2 kg, all fish sampled were male (40 % in stage M2 and 60 % in stage M3). At weights between 2–2.5 kg, the proportion of males in the sample rapidly decreased from 100 % as seen in fish <2 kg, to 62.5 %; approximately 12.5 % of the sample was classified as exhibiting male to female gonadal transition, and the remaining 25 % completed feminization. Some of the females in the 2–2.5 kg weight class exhibited relatively advanced F3 vitellogenic gonadal status. One notable case was a female undergoing folliculogenesis at 2.06 kg. In the 2.5–3.0 kg weight category, the proportion of males had further reduced to comprise <40 % of the fish sampled, with approximately 25 % of these males exhibiting spermatogenesis (M2) and 12 % in the post-spawning (M4) stage. In the same weight category, 37.5 % of barramundi were transitioning to female (T2, T3, and T4 stages). At weights of 3.0–3.5 kg, 65 % of the sample were female and in 5% of these females evidence of recent ovulation, or follicular involution, was observed with gonads exhibiting

oocyte atresia (F4). Transitional fish represented only 5% of the sample in the 3.0–3.5 kg weight category and all were in the final stages of gonadal transition (T4). Between 3.5–7.0 kg, the proportion of females continued to increase. Above 7.0 kg, all fish in the sample were female.

To better understand the sex change process in relation to fish size, bivariate linear regressions of total body length (in 5 cm intervals), and body weight (in 500 g intervals) of males vs. transitional and/or females was used to enable estimation of the proportion of the population that had initiated, or completed the sex change process at any given size (Fig. 2A). At 55 cm and below, approximately 35 % of the fish sampled had already initiated, or completed sex change. The fish body size at which 50 % of barramundi had transitional or female gonads was 59.0 cm and 2.68 kg. Between 60–70 cm, 60–65 % of the population were transitioning, or had completed the sex change process to be female. In fish 70–80 cm in length, approximately 85–95 % of the fish had completed, or were in the process of changing sex. At a length greater than 85 cm, no fish had visible gonadal testicular tissues.

Gonadosomatic index (GSI) of the sampled barramundi were plotted according to their respective gonad maturation stages (Fig. 2B). In general, mean GSI values for males were the lowest ($GSI = 0.41 \pm 0.24$), followed by transitional staged fish ($GSI = 0.54 \pm 0.64$). Females as expected exhibited highest GSI values ($GSI = 1.60 \pm 1.56$). GSI values for males, transitional fish, and F1/F2 staged females remained below 2. Within fish classified as male, M2 fish exhibited the highest GSI values, with the maximum GSI being 1.04. GSI values for transitional fish were also found to remain below 2 (with one exception reaching 2.6), where T1 and T4 staged fish were low (<1) and T2 and T3 staged fish had slightly higher values. F3 females had the largest ovaries and therefore the highest GSI values, with the greatest distribution as compared to fish of any other gonad maturation stage ($GSI = 2.70 \pm 1.65$). F4 females were found to have the next highest GSI values and distribution with a mean value of 1.78 ± 1.07 (Table 2, Fig. 2B).

A small percentage of the sampled fish (3%) were found to contain gonads that had intersex characteristics, possessing gonads with both ovarian (active folliculogenesis – F3) and testicular (active spermatogenesis –M2, or spermiation M3) tissues. These animals were classified as T2 transitional fish, as both ovarian and testicular tissues were present as described by Guiguen et al. (1994) (Table 1). Active spermatogenesis was sometimes observed adjacent active folliculogenesis (Fig. 3, A–D). In some fish, a whole range of developing stages, ranging from previtellogenic to vitellogenic oocytes, spermatocytes, spermatids, and spermatozoa could be observed.

To establish if barramundi reared in Singapore exhibited a different pattern of sex differentiation than that described elsewhere, data from sea-cage reared barramundi were compared to that of sea-cage farmed fish in French Polynesia (Guiguen et al., 1994) (Fig. 4). At body weights lower than 2.5 kg, in French Polynesia all fish were still male; whereas approximately 25 % of Singapore barramundi were already female. Females first appeared in the French Polynesian sample from 2.5 to 3.0 kg in weight, accounting for 15 % of the population; while in Singapore, the proportion of females was more than double (33 %). Barramundi were observed to be transitioning in Singapore-farmed fish from ~ 2.0–2.5 kg, always remaining less than 20 % of the total distribution (except for the 7.5–8.0 kg weight category where 40 % of the fish were transitioning). In contrast, French Polynesian transitional fish were observed in a narrower size window (3.5–6.0 kg), remaining in proportions of <20 % of the fish in each category. Comparing the weight at which the proportion of males became ≤50 % of the sampled population, this weight was at 2.5–3.0 kg in Singaporean barramundi and 5.0–5.5 kg in French Polynesia. By comparing the increase in percentage of females post sex change, it was observed that in Singaporean barramundi 70 % of the fish were female at 3.0–3.5 kg, 90 % were female at 5.0–5.5 kg, and 100 % were female at 6.5–7.0 kg. In French Polynesia, it was not until 6.0–6.5 kg that ~70 % of the fish were female. This highlights that barramundi in Singapore appear to be initiating the sex-change process at smaller sizes than those reported in French

Table 2

Summary statistics (mean \pm S.D. (minimum – maximum) values) of body weight, total length, body depth, body width, Fulton’s condition factor (K), body shape (H), and gonadosomatic index (GSI) in barramundi, classified by histology as either male, transitional, or female. Different superscript letters denote statistically significant differences among male, transitional and female fish (ANOVA and Tukey’s HSD, $P < 0.05$).

Sex type	n	Body weight (Kg)	Total length (cm)	Body depth (cm)	Body width (cm)	Fulton’s condition factor (K)	Body shape (H)	GSI
Males	31	3.39 \pm 1.27 (1.26–6.46) ^a	63.6 \pm 7.7 (46–76) ^a	17.1 \pm 3.1 (10.7–23.3) ^a	6.29 \pm 1.09 (4.73–9.02) ^b	1.26 \pm 0.13 (0.99–1.52) ^a	2.68 \pm 0.26 (2.25–3.28) ^a	0.41 \pm 0.24 (0.11–1.04) ^a
M1	1	2.26	56	14.5	5.7	1.29	2.59	0.22
M2	14	3.49 \pm 1.09 (1.84–5.22)	65.0 \pm 7.0 (54–74)	17.7 \pm 3.0 (12.4–23.3)	6.29 \pm 0.87 (4.73–7.67)	1.24 \pm 0.13 (1.08–1.52)	2.72 \pm 0.27 (2.25–3.28)	0.49 \pm 0.26 (0.13–1.04)
M3	15	3.40 \pm 1.50 (1.26–6.46)	62.9 \pm 8.6 (46–76)	16.8 \pm 3.4 (10.7–22.9)	6.42 \pm 1.28 (4.95–9.02)	1.28 \pm 1.48 (0.99–1.47)	2.66 \pm 0.26 (2.34–3.23)	0.38 \pm 0.21 (0.11–0.94)
M4	1	2.96	62	15.5	4.86	1.24	2.5	0.17
Transitional	16	4.19 \pm 1.25 (2.24–6.48) ^a	67.5 \pm 7.6 (52–78) ^a	19.2 \pm 3.6 (12.4–25.6) ^a	7.31 \pm 1.25 (5.49–8.98) ^a	1.33 \pm 0.16 (0.99–1.64) ^a	2.82 \pm 0.30 (2.40–3.41) ^a	0.54 \pm 0.64 (0.02–2.60) ^a
T1	1	5.02	72.8	22.8	8.98	1.3	3.14	0.02
T2	9	4.36 \pm 1.35 (2.24–6.48)	68.0 \pm 8.7 (52–78)	18.9 \pm 4.2 (12.4–25.6)	7.30 \pm 1.36 (5.49–8.91)	1.36 \pm 0.16 (1.16–1.64)	2.76 \pm 0.36 (2.40–3.41)	0.73 \pm 0.78 (0.15–2.60)
T3	3	4.23 \pm 1.47 (2.62–5.48)	66.9 \pm 8.4 (58–74)	19.0 \pm 3.7 (16.0–23.2)	7.19 \pm 1.32 (6.18–8.69)	1.37 \pm 0.24 (1.35–1.40)	2.84 \pm 0.27 (2.61–3.13)	0.49 \pm 0.41 (0.18–0.95)
T4	3	3.35 \pm 0.88 (2.66–4.34)	64.9 \pm 6.0 (59–71)	18.8 \pm 2.9 (15.8–21.7)	6.90 \pm 0.96 (6.10–7.96)	1.23 \pm 0.25 (0.99–1.49)	2.88 \pm 0.19 (2.68–3.06)	0.22 \pm 0.13 (0.07–0.30)
Females	126	5.12 \pm 1.22 (2.06–7.62) ^b	72.4 \pm 5.8 (49–87) ^b	21.7 \pm 3.0 (14.2–26.5) ^b	7.81 \pm 1.17 (5.36–10.58) ^a	1.33 \pm 0.14 (0.99–1.81) ^a	2.98 \pm 0.27 (2.10–3.62) ^a	1.60 \pm 1.56 (0.04–7.58) ^b
F1	32	4.78 \pm 1.07 (3.12–6.88)	72.0 \pm 5.3 (64–83)	21.4 \pm 2.3 (16.5–25.8)	7.80 \pm 1.03 (5.36–9.87)	1.27 \pm 0.12 (1.05–1.54)	2.97 \pm 0.22 (2.54–3.53)	0.35 \pm 0.21 (0.04–0.86)
F2	25	5.39 \pm 1.28 (2.14–7.62)	74.1 \pm 5.5 (60–87)	22.8 \pm 2.3 (16.4–25.9)	8.23 \pm 1.40 (5.56–10.58)	1.30 \pm 0.14 (0.99–1.73)	3.07 \pm 0.20 (2.69–3.45)	0.53 \pm 0.32 (0.13–1.29)
F3	59	5.19 \pm 1.24 (2.06–7.58)	72.0 \pm 6.2 (49–82)	21.4 \pm 3.5 (14.2–26.5)	7.68 \pm 1.09 (5.54–10.38)	1.37 \pm 0.14 (1.04–1.81)	2.97 \pm 0.32 (2.10–3.62)	2.70 \pm 1.56 (0.44–7.58)
F4	10	5.06 \pm 1.33 (3.48–7.62)	71.9 \pm 5.7 (64–78)	21.0 \pm 3.3 (16.7–25.2)	7.58 \pm 1.35 (5.61–8.97)	1.34 \pm 0.11 (1.19–1.62)	2.91 \pm 0.26 (2.63–3.28)	1.78 \pm 1.07 (0.34–3.71)
Total/ average	173	4.72 \pm 1.40 (1.26–7.62)	70.4 \pm 7.2 (46–87)	20.6 \pm 3.6 (10.7–26.5)	7.49 \pm 1.30 (4.73–10.58)	1.32 \pm 0.14 (0.99–1.81)	2.91 \pm 0.30 (2.10–3.62)	1.29 \pm 1.44 (0.02–7.58)

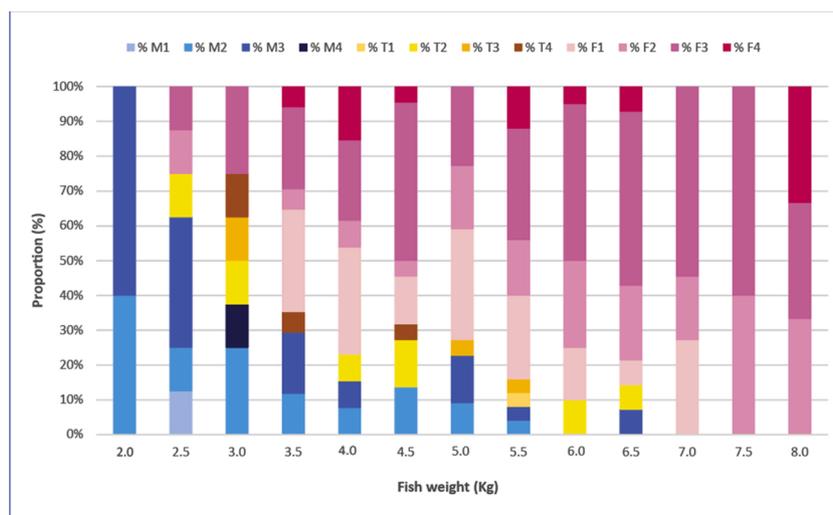


Fig. 1. Distribution of gonadal sex (M = male, T = transitional stage, and F = female) and gonad developmental stages (M1 – M4 = testis stage 1 – 4, T1 – T4 = transitional gonad stage 1 – 4, F1 – F4 = ovary stage 1 – 4) in barramundi sampled from sea cages at 0.5 kg body weight intervals (*2 kg = 1.51 kg to 2.0 kg, etc.).

Polynesia.

4. Discussion

The present study supports anecdotal reports that barramundi farmed in Singapore were undergoing sex change from male to female earlier than has been previously documented in Australia, French Polynesia and other parts of Southeast Asia. Histological analysis of gonadal tissues in 2.5 year old fish, showed that nearly 75 % of fish were phenotypic females, 10 % were transitioning to female and less than 15 % were phenotypic males. Females were significantly larger and heavier

(5.12 \pm 1.22 kg; 72.4 \pm 5.8 cm) than males (3.39 \pm 1.27 kg; 63.6 \pm 7.7 cm), indicating that faster growth is associated with sex change. Using a different approach whereby otolith microstructure was analysed to explore growth throughout ontogeny in a wild barramundi population, Roberts et al. (2021) observed that growth rate influences age at sex change, with faster growing individuals changing sex at younger age than those that grew slower. In contrast to our findings though, Roberts et al. (2021) considered barramundi females less than 5 years of age to have transitioned sex when young, and females smaller than 85 cm were considered small-sized during sex change. While the causes for such precocious transitioning of sex are unclear, there are

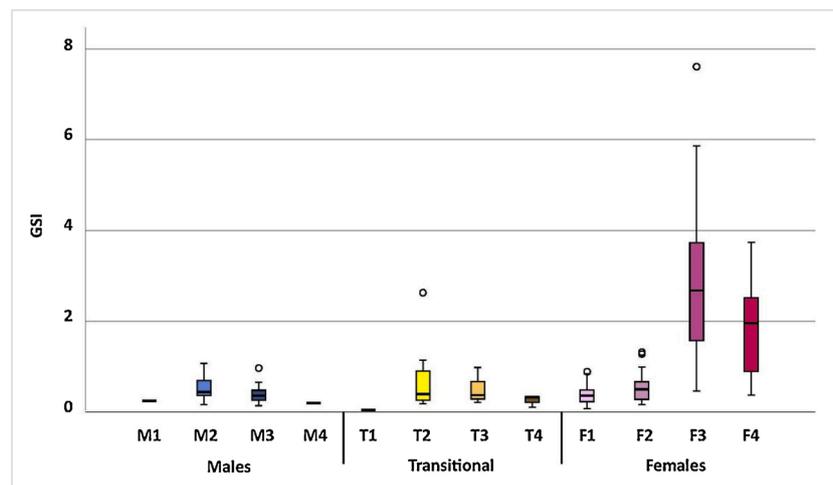
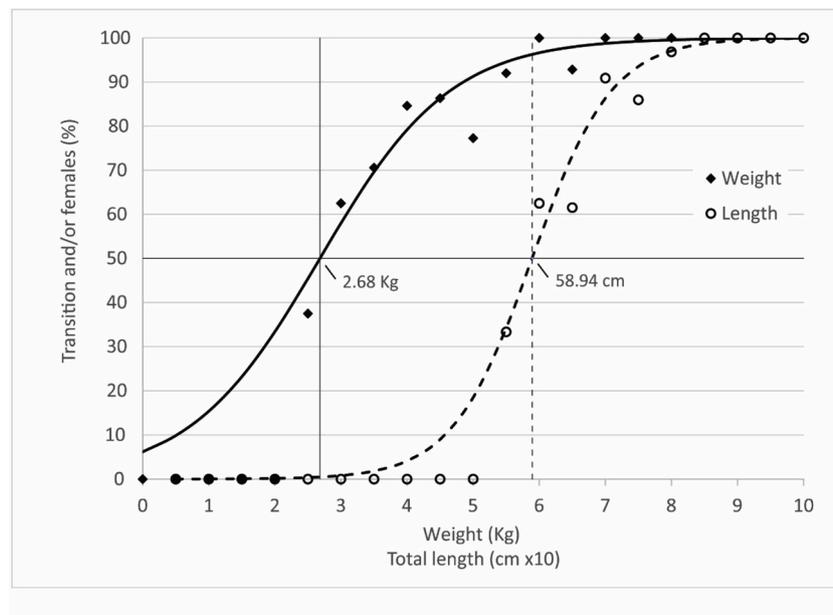


Fig. 2. (A) Proportion of barramundi farmed in Singapore having initiated or completed the sex change process (i.e. fish classified with transitional and/or female stage gonads) according to total length and weight. (B) Distribution of gonadosomatic index (GSI) according to gonadal maturation stages for males (M1 – M4), transitional fish (T1 – T4), and females (F1 – F4).

important consequences of these observations on the dynamics of captive breeding programs in Singapore. The short transience of reproductively active male individuals before they change sex to female could represent a challenge for maintaining an optimal male to female ratio of 2:1 in barramundi breeding populations (Toledo et al., 1991; Lim et al., 1986). Observations from the present study indicate that selection and retention of broodstock candidates older than two years of age would likely be too late to ensure sufficient numbers of reproductive males. Selection for broodstock candidates is usually based on size (length or weight). Results of this study indicate that when selecting fish of 59 cm L_t (or 2.7 kg total body weight), less than 50 % may be male and in fish of 65 cm L_t (or 3 kg total body weight), the proportion of males drops dramatically to only 30 % of the population. At two and a half years of age, 55 % of fish identified as females in this study were already reproductively mature and with ovarian development indicative of being in spawning condition. In the mid-1980s, Lim et al. (1986) described female barramundi cultured in Singapore attaining maturity at ~3–4 years and spawning at body weights ranging from 3 to 12 kg. In French Polynesia, male to female sex change of barramundi reared in sea cages was reported to take place at 3–5 years of age (Guiguen et al.,

1994). In comparison, barramundi farmed in Singapore in the present study were found to be changing sex at half the weight of what was reported in French Polynesia (5 kg) (Guiguen et al., 1994).

In the protandrous barramundi, testicular differentiation begins at around 44 days post hatch (dph), with gonadal development proceeding from the anterior to the posterior portion of the tissue, and testes being fully formed around 140 dph (Banh et al., 2017). In Singapore, active spermatogenesis was observed in approximately half of the fish identified as males, with one fish from this batch (out of 31) showing evidence of having recently spawned. In Australian wild stocks, males are known to attain sexual maturity in their 3rd–5th year, measuring 55–60 cm total length, before initiating sex change after an average of 5 years of age (Davis, 1982). The testes mature for a final round of spawning, before a month-long transition to an ovary is completed (Davis, 1982). The speed of transition and maturation of such young females was likewise not previously reported. Most reports investigating protandry within the species have targeted wild populations in Australia, Asia, and other regions, where barramundi can be found; nevertheless, such studies have described a much delayed sexual maturation and transition than what has been described here. For instance, in a recent study,

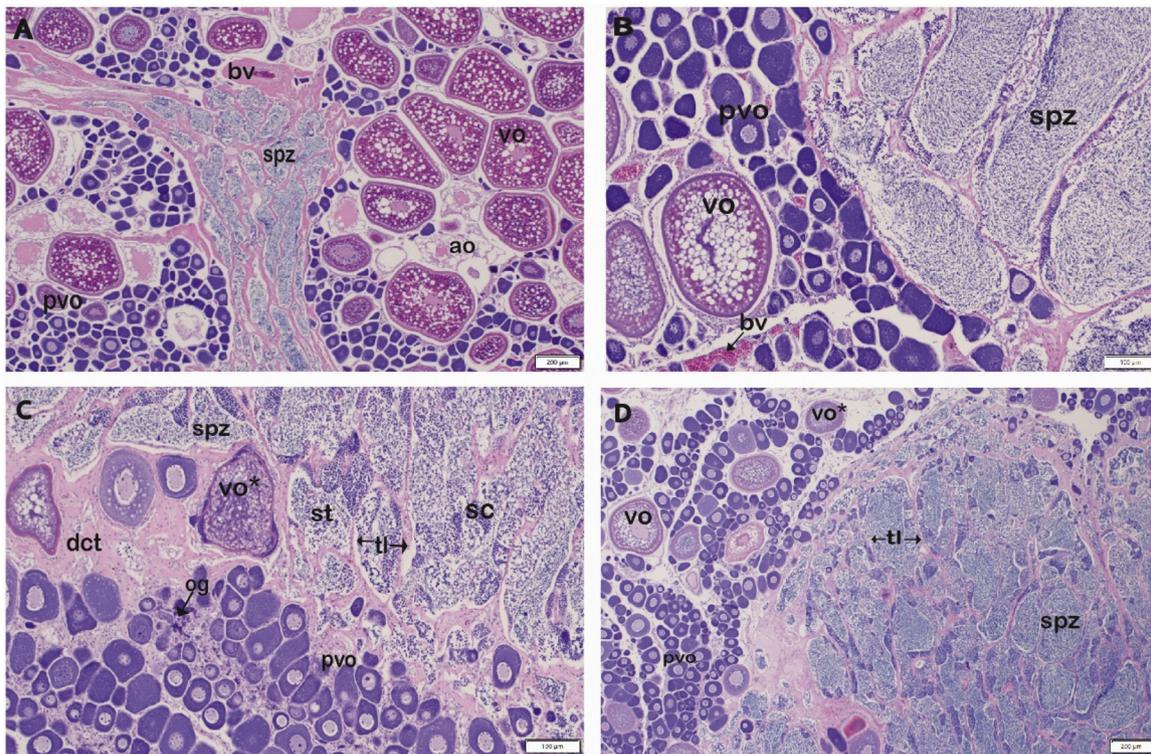


Fig. 3. Cross sections of gonads in different fish with intersex characteristics in barramundi, (H&E staining) in which testicular tissue consisting of spermatozoa were embedded within (A) or adjacent to (B-D) ovarian tissue consisting of oocytes in varied developmental stages. Barramundi from which gonads were sampled and shown in the figures, weighed 3.5 kg (A), 2.9 kg (B) and 4.2 kg (C and D). st = spermatids, sc = spermatocytes, spz = spermatozoa, tl = testicular lobules, pvo = previtellogenic oocytes, vo = vitellogenic oocytes, ao = atretic oocytes, og = oogonia, and dct = dense connective tissue, and vo* = early stage vitellogenic oocyte.

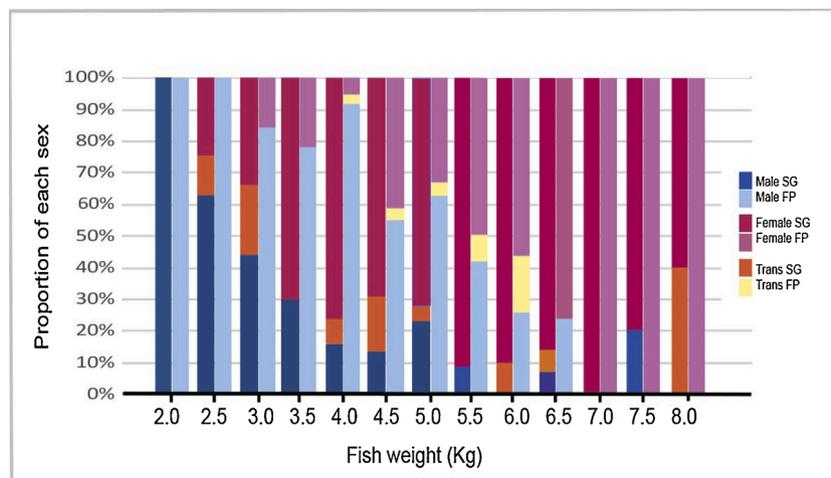


Fig. 4. Distribution of Barramundi, males, females, and transitional (Trans) gonadal sex types at 500 g body weight intervals* of a population farmed in Singapore (SG) and in French Polynesia (FP); adapted from Guiguen et al. (1994). *2.5 kg = 2.01 to 2.50 kg, etc.

Roberts et al. (2021) found that the age and size in which a barramundi population from the Fitzroy catchment of northern Western Australia attains a sex ratio of 1:1 is around 6.85 years old and 92.7 cm. In western Papua, females were not found in the population until 5 years of age and 73 cm total length, with the exception of one 2-year old female (Moore, 1979). Mature males were first observed at 46 cm in the present study, which is a smaller size than that reported by Davis (1982) at 60 cm and 55 cm in the Northern Territory and Gulf of Carpentaria, respectively.

A high percentage (9.2 %) of the Singaporean farmed population examined in our study possessed transitional gonads, compared to 1.4 %

of 432 fish in western Papua, 3.2 % of 304 fish sampled in Northern Territory and 9% of 322 fish sampled in the Gulf of Carpentaria, Australia (Moore, 1979; Davis, 1982). Interestingly herein, three different transitional stages were observed in seemingly equal proportions for fish weighing between 3.0 and 3.5 kg. At 3.5 kg or more, only a small proportion of fish were observed in the final transitional stage, contributing to the body of evidence reporting how rapid the sex change process occurs in barramundi (Moore, 1979; Guiguen et al., 1994). Transitional fish in the current study measured 67.5 cm (mean length), which is significantly smaller in comparison to those reported

by Davis (1982) for the Northern Territory at 90.7 cm (at 5.4 years of age) and the Gulf of Carpentaria at 82.0 cm in mean length (at 5.3 years of age), respectively. Slight differences in length during sex change among populations have also been recently reported. Comprehensive data from the Queensland Department of Agriculture and Fisheries (DAF) Long Term Monitoring Program (LTMP - 2000–2016) of barramundi sampled from the North Queensland East coast ($n = 1999$) and the Southern Gulf of Carpentaria ($n = 4871$) estimated that the size in which 50 % of these two populations were female was around 92 cm, and for the mid-Northern Gulf of Carpentaria fishery ($n = 4900$) this size was somewhat comparatively smaller, around 77 cm (Budd, 2020). Within that same geography, but north of latitude S 13° within the Gulf of Carpentaria, Davis (1984) had previously reported one unusually precocious population of *L. calcarifer*. In that population, males were found to sexually mature at 1–2 years of age (and no males longer than 50 cm); at a length of 45.5 cm, half of the surveyed individuals were females. However, different from what was found in the farmed individuals in the present study, severe stunting was found in that population, with mean length of 4 year old fish measuring approximately 40 cm. Despite the small size of females reported by Davis (1984), with >20 % of 1–2 year old fish being female, most sex change occurred between 3 and 4 years of age.

Environmental factors can influence sex in fish in addition to genetic factors. Where environmental factors are concerned, temperature is the best-known sex determinant, especially in species that exhibit temperature dependent sex determination (TSD) (Devlin and Nagahama, 2002; Martínez et al., 2014; Budd et al., 2015). Temperature is also a factor influencing not only growth of barramundi, but also the regulation of the speed of sex change (Athauda et al., 2011; Athauda and Anderson, 2014; Budd, 2020). After holding 15 month-old males at temperatures ranging from 22 to 34 °C for 14 weeks, Athauda and Anderson (2014) observed that plasma sex steroid levels and aromatase activity in barramundi increased with increasing temperature, with a greater number of fish entering transitional stages at 31 °C and 34 °C. More recently, Budd (2020) confirmed similar effects when observing that 80 % (4/5) of barramundi held from 12 to 24 months-post-hatch (mph) at 34 °C were transitioning, or female, as opposed to 0% (0/5) of fish held at 24 °C. Budd (2020) also demonstrated that temperature-induced gonadal changes were accompanied by epigenetic and transcriptional changes in key sex genes, such as *cyp19a1* and *dmrt1*, reflecting the patterns reported for wild and cultured adult males and females of the species (Domingos et al., 2018). Considering Singapore's location near the equator (N 1°11' ~ N 1°28'), water temperatures are warm (28–31 °C) all year round and reportedly rising by 0.25 °C per decade since 1948 (Chou and Lee, 1997; Meteorological Services Singapore, www.weather.gov.sg/climate-past-climate-trends/). Temperature effects may be one cause for such a difference in barramundi maturity and early sex change observed here in comparison with previous observations made in Singapore, Australia, French Polynesia, and western Papua. Apart from the barramundi, other fish species have also exhibited sensitivity to temperature in terms of growth and their sexual differentiation. The protogynous hermaphrodite black seabass (*Centropristis striata*) has shown varied patterns of sexual differentiation when cultured in varying water temperatures, where the percentage of fish that matured as females reduced as temperatures decreased, implying that low temperatures might retard or delay the sexual development process in the species (Colburn et al., 2009). These observations reiterate the influence of temperature on sex change and reproduction. Although temperature may play a key role in the early sex change, it may not be the only factor influencing the process. Sex change in protandrous hermaphrodites, like barramundi, could further be influenced by an array of factors that result from the unique farming environments they are reared under, such as artificial social structures, nutrition, husbandry, mortality rates and growth rates (Munday et al., 2006).

An unusually high proportion of barramundi individuals (5 out of

173) found in the present study were synchronous hermaphrodites, or intersex, with a different morphology than that expected for transitional staged gonads. These fish possessed gonads with both mature vitellogenic oocytes and spermatozoa in well-developed testicular lobules with tissues of neither sex being in a regressed, or degrading condition. Such abnormal hermaphrodite gonads had been previously described by Moore (1979) and Davis (1982), but at much lower frequencies of 0.01 % (5 out of 5202) and 0.34 % (3 out of 880), respectively. Synchronous hermaphroditism, or the intersex condition, is a phenomenon yet to be understood in the species. However, this abnormality associated with such early sex change in this population raises questions as to whether the temperature of Singaporean waters is the sole driving factor. Other factors such as water pollution brought about by the surrounding maritime traffic and activities of the nearby petrochemical hub may have a role in how soon the barramundi matures. A similar instance of intersex gonads found in cobia (*Rachycentron canadum*) was reported by Dutney et al. (2017), where the naturally gonochoristic species was observed to have a high percentage (16.9 % and 6.8 %) of fish being intersex after periods of heavy flooding carried urban and agricultural run-off to the adjacent coastal area where water was sourced. The intersex fish observed in the current study had gonadal structures that were largely similar to those described by Dutney et al. (2017), where the proportion of testicular and ovarian tissue varied between the individuals independent of the gonad size. The presence of a distinct junction between the ovary and testis section containing connective tissue was also similar in both species cases. In another fish, roach (*Rutilus rutilus*), intersex individuals were found in wild populations and linked to feminizing chemical agents in the water (Jobling et al., 2002a). Environmental pollutants may contain endocrine disrupting chemicals (EDC) which can interfere with the function of endogenous hormones in the animals, resulting in abnormalities in their reproductive development (Jobling et al., 2002b; Bergman et al., 2012; Purdom et al., 1994). Dutney et al. (2017) noted that the intersex fish observed in their study could be males that were under a de-masculinising, or feminizing influence from the environmental pollutants. Although the percentage of intersex fish in this study were low when compared to the work of Dutney et al. (2017), this incidence of intersex barramundi only represents the sample size collected from a single farm in Singapore. It is yet undetermined if similar occurrences can be observed in wild populations of barramundi, or in other farms in Singapore. Singapore is one of the busiest ports in the world, with more than 130,000 vessel calls annually (Maritime and Port Authority of Singapore, www.mpa.gov.sg/web/portal/home/maritime-singapore/introduction-to-maritime-singapore/pr-emier-hub-port). The constant movement of large shipping vessels, potential vessel discharges and oil spills, and petrochemical plants located on offshore islands may also be a source of xenobiotics which might disrupt the synthesis and metabolism of sex hormones in fish that are farmed in local waters; leading to such abnormal intersex conditions in an otherwise sequentially hermaphroditic species. Although this study has not investigated potential factors, the hepatic transcriptome of barramundi sampled from three agriculturally impacted rivers in Queensland, Australia, has shown alterations within xenobiotic metabolism, peroxisome proliferation, stress responses, and reproductive function (Hook et al., 2017).

5. Conclusions

This study reports the occurrence of precocious sex change of sea-cage farmed *L. calcarifer* in Singapore. Results suggest that the selection of broodstock candidates before two years of age is required to ensure the best chance of acquiring sufficient males for breeding before they begin to sex transition to female. The rapid period of transition and maturation of females that was observed would mean that farmers need to be prompt, consistent, and diligent when selecting and managing their broodstock in order to maintain the desired 2:1 male-to-female ratio. The causes for the early sex change are yet unknown and

deserve further investigation. Further studies examining the sex change patterns of barramundi from a wider spread of samples of varied body sizes and from other farms are warranted and would add value to the current work. Environmental assessments that examine the sexual development of barramundi in other surrounding regions may point the way forward in determining the causes for the phenomenon observed in this study. Furthermore, although early sex change has been noted for Singapore-farmed barramundi, the effects it has on the physiology of the gonads and quality of gametes produced have yet to be determined.

CRediT authorship contribution statement

Celestine Terence: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Jose A. Domingos:** Conceptualization, Investigation, Formal analysis, Project administration, Writing - review & editing. **Joseph Angelo Uichanco:** Investigation, Resources, Writing - review & editing. **Yann Monteil:** Conceptualization, Writing - review & editing. **Loïc Monteil:** Conceptualization, Writing - review & editing. **Emmanuel De Braux:** Resources, Writing - review & editing. **Quyen Q. T. Banh:** Formal analysis, Writing - review & editing. **Susan Gibson-Kueh:** Formal analysis, Writing - review & editing. **Dean R. Jerry:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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