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Original Article

Cyp19 expression and sex change timing in captive-bred false clownfish

Expressão de *Cyp19* e o período da mudança de sexo em peixes-palhaço falsos criados em cativeiro

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Abstract

The false clown anemonefish (*Amphiprion ocellaris*) is a protandrous hermaphrodite with a distinctive reproductive behavior. This study elucidates the genetic mechanisms and timing of sex changes in captive-bred *A. ocellaris* by examining the expression of key genes involved in this process, specifically *cyp19a1a* and *cyp19a1b*. Gonadal histological analyses and gene expression studies were conducted on subadult fish paired for 0, 1, 2, 3, 4, 5, and 16 months. Our findings reveal that alterations in *cyp19* gene expression coincide with a pairing period starting after 3 months. Both *cyp19a1a* and *cyp19a1b* expression levels were significantly elevated in paired females compared with their male counterparts and unpaired controls. Histological investigations demonstrated that sex conversion to females occurred during the 3-month pairing period. This study highlights the crucial role of *cyp19a1a* and *cyp19a1b* in the sex change process of *A. ocellaris* and indicates that a minimum of 5 months of pairing is necessary for completing the sex change.

Keywords: anemonefish, clownfish, *cyp19a1a*, *cyp19a1b*, sex change.

Resumo

O peixe-palhaço falso (*Amphiprion ocellaris*) é um hermafrodita protândrico com um comportamento reprodutivo distintivo. Este estudo esclarece os mecanismos genéticos e o período das mudanças de sexo em *A. ocellaris* criados em cativeiro, examinando a expressão de genes-chave envolvidos nesse processo, especificamente *cyp19a1a* e *cyp19a1b*. Análises histológicas gonadais e estudos de expressão gênica foram conduzidos em peixes subadultos emparelhados por 0, 1, 2, 3, 4, 5 e 16 meses. Os resultados revelam que alterações na expressão do gene *cyp19* coincidem com um período de emparelhamento a partir de 3 meses. Os níveis de expressão de *cyp19a1a* e *cyp19a1b* foram significativamente elevados em fêmeas emparelhadas em comparação com seus homólogos masculinos e controles não-emparelhados. Investigações histológicas demonstraram que a conversão sexual para fêmeas ocorreu durante o período de emparelhamento de 3 meses. Este estudo destaca o papel crucial de *cyp19a1a* e *cyp19a1b* no processo de mudança de sexo de *A. ocellaris* e indica que um mínimo de 5 meses de emparelhamento é necessário para concluir a mudança de sexo.

Palavras-chave: peixe-anêmona, peixe-palhaço, *cyp19a1a*, *cyp19a1b*, mudança de sexo.

1. Introduction

The false clown anemonefish, *Amphiprion ocellaris* Cuvier (1830), is a captivating marine species known for its unique reproductive behavior. As a protandrous hermaphrodite, it initially develops as a male before transitioning to a female later in life, positioning it as a valuable model for investigating sex changes in fish (Godwin, 2010). In their natural habitat, these anemonefish form social groups comprising a dominant female, a male partner, and smaller, immature males (Buston, 2003). Upon the leading female's death or removal, the most prominent male typically undergoes a sex change, with the remaining males progressing in the hierarchy (Fricke and Fricke, 1977). Body size is a proxy for social rank, indirectly influencing the change. However, the genetic factors prompting sex change in the false clown anemonefish remain elusive, particularly concerning process initiation, gonadal transformation duration, and specific gene involvement (Fricke and Fricke, 1977; Godwin, 2010).

Several sex-related genes, such as *Sox* (SRY-related genes with an HMG box), *amh* (anti-Müllerian hormone), and *cyp19* (aromatase (P450arom)), have been implicated in sex change in various fish species (Devlin and Nagahama, 2002; Kobayashi et al., 2010; Guiguen et al., 2010). The *cyp19* gene, encoding the aromatase enzyme complex, is particularly intriguing due to its critical role in maintaining sex steroid hormone balance by converting androgen male sex hormones into estrogen. Fish exhibit two distinct CYP19 protein isoforms—P450aromA and P450aromB—products of the *cyp19a1a* (*cyp19a*) and *cyp19a1b* (*cyp19b*) loci, respectively.

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Cyp19a1a is primarily expressed in female gonads, influencing ovarian differentiation and maturation, whereas *cyp19a1b* is predominantly expressed in female brains, impacting reproductive behavior and sex change in some species (Kishida and Callard, 2001).

This study's primary objective is to elucidate the timing and genetic mechanisms underpinning sex change in the false clown anemonefish. We specifically examined the expression levels of essential genes involved in reproductive development, such as the ovarian aromatase *cyp19a1a* gene in the gonadal tissue and the aromatase *cyp19a1b* gene in the brain, throughout the sex change process in paired subadults under controlled rearing conditions. To minimize age and body-size biases, our initial experiment involved selecting 8-month-old fish from the same parents with negligible size and genetic differences. We identified each couple's male or female status by investigating the sex change process onset in the subadult clownfish, considering that females typically exhibit faster growth than males (Buston, 2003; Munday et al., 2006). Consequently, we measured specific growth rates (SGR) as a percent-length increase per day. Furthermore, we assessed the duration of gonadal transformation and the involvement of particular genetic factors, analyzed *cyp19a1a* and *cyp19a1b* gene expression patterns during various sex change stages, and conducted histological analyses to determine gonadal development progression throughout the sex change process. The outcomes of this study will not only enhance our understanding of the genetic factors driving sex change in the false clown anemonefish but also optimize captive breeding programs and promote the conservation and sustainability of this emblematic species.

2. Materials and Methods

2.1. Experimental fish and growth measurement

Experimental fish were propagated from a single pair of breeders obtained from the Samut Sakhon Coastal Fisheries Research and Development Center. Parents were reared in a 120-L fiberglass tank at the Wet laboratory in the Department of Aquatic Science, Faculty of Science, Burapha University, under controlled environmental parameters. The fish were fed twice daily with commercial food (NRD G8, INVE Aquaculture, Thailand). The experimental design is illustrated in Figure 1. Post-hatching, 200 fries were reared for a month and divided into two tanks with 46 fish each until they reached eight months. The remaining fish with similar size (in total length; *Lt*) were pooled and randomly divided into two groups: an unpaired control group (n = 28, *Lt* = 49.97 ± 0.40 mm) and a paired subject group (n = 54, *Lt* = 49.88 ± 0.87 mm). The control group was divided among seven separate 120-L tanks, with four fish housed per tank. Each fish was separated using circular plastic netting (9-mm mesh diameter) to maintain a minimum distance of 5 cm between them. From 8 to 13 months of age and again at 24 months, fish in each tank was sampled monthly for growth rate measurements and further analysis.

For the paired subject group, a new set of four pairs was chosen for each sampling event. Sample collection resumed 16 months after pairing, but only three fish pairs were used for this sampling event. The gonadal tissue was removed from each fish, and the left half was used for RNA extraction and subsequent gene expression analysis. Simultaneously, the brain tissue was also collected for gene expression analysis.

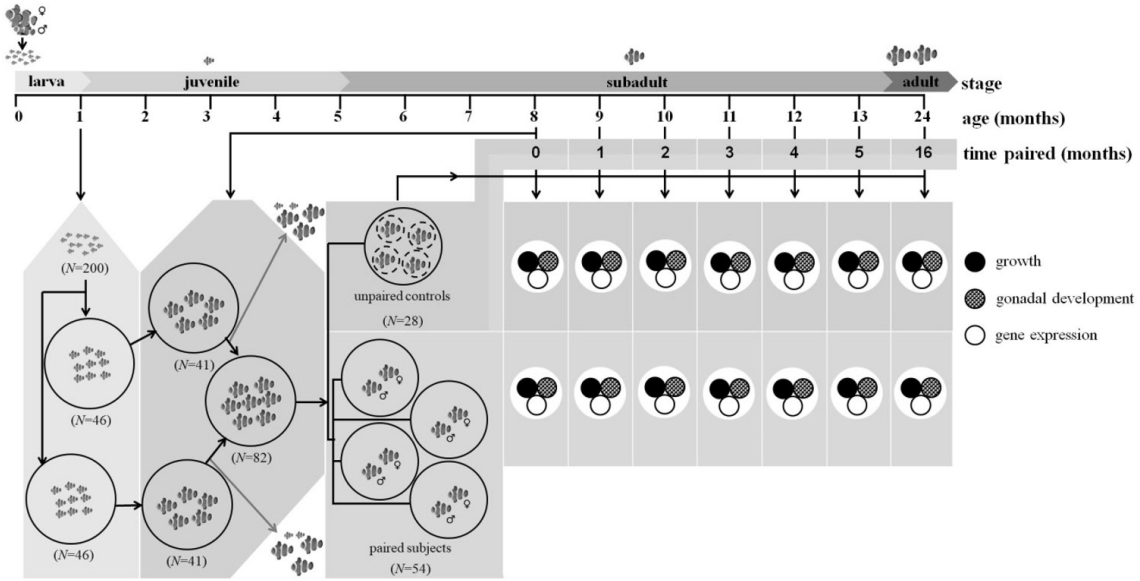

Figure 1. Experimental design for fish sampling and analysis. Two hundred *Amphiprion ocellaris* fries were raised for a month and divided into two tanks. At 8 months, fish were randomly assigned to unpaired control (n = 28) and paired subject (n = 54) groups. Control fish were separated into 7 aquaria, with 4 fish per tank separated by plastic netting. Fish were sampled monthly from 8 to 13 months and at 24 months for growth measurement and analysis. For the paired subject group, 4 new pairs were chosen per sampling event, with sample collection resuming at 16 months. Gonadal and brain tissues were extracted for gene expression analysis and histological examination.

The remaining half of the gonadal tissue was specifically reserved for histological examination. Fish were fed three times daily after hatching, and the feeding frequency was reduced to twice daily from the age of three weeks. Commercial pellets were provided twice daily as the primary food source from four weeks of age. Daily fish health, feeding, and tank maintenance monitoring were conducted while culture conditions remained consistent with the parent stock. Each fish was euthanized after rearing at the desired age, and the total length (L_t in mm) was measured. The specific growth rate (SGR) was determined using Formula 1 (Lugert et al., 2016):

$$SGR\ \left(\%\ day^{-1}\right) = \left[\left(Log\ Final\ L_t - Log\ Initial\ L_t\right)\ /\ Number\ of\ days\right] x\ 100 \quad (1)$$

All animal care and experimentation followed Burapha University guidelines (Approval ID # IACUC 040-2561).

2.2. Histological analysis of gonadal development

The gonads were fixed in Bouin's solution and prepared for histological analysis. After fixation, the gonads were dehydrated, cleared, and embedded in paraffin blocks. Longitudinal serial sections were cut at 5 µm intervals and stained with Harris hematoxylin and eosin. The sections were examined under a microscope to determine the developmental stage of the gonads based on morphological characteristics, following the references by Parenti and Grier (2004) and Dietrich and Krieger (2009). Images were acquired using a DP22 digital camera (Olympus), and the proportion of the area occupied by ovarian and testicular cells in the gonad sections was scored using Olympus Stream image analysis software (Olympus) and ImageJ software v2.0 (NIH, Bethesda, MD, USA).

2.3. Total RNA isolation and cDNA synthesis

The gonadal tissue was used to analyze *cyp19a1a* expression, while the brain tissue was utilized to detect *cyp19a1b* expression. Total RNA was extracted from target tissues using TRIzol™ reagent (Invitrogen, USA) following the manufacturer's protocol. The purity and concentration of RNA were determined using a spectrophotometer (NanoDrop, Thermofisher), with $A_{260/280}$ values around 2.0. A DNase treatment was performed to remove any genomic DNA contamination, and the RNA was stored at -80°C until further use. Following the manufacturer's instructions, one microgram of RNA was used for cDNA synthesis in a 20 µl using the Applied Biosystems™ High Capacity cDNA reverse transcription kit and 200 ng of random hexamer primers (Applied Biosystems™, USA).

2.4. Quantitative real-time PCR (qRT–PCR)

Primers specific for *cyp19a1a* and *cyp19a1b*, and the internal control gene, *18S rRNA*, were designed for qRT–PCR reaction based on mRNA sequences from the NCBI GenBank database. Primer3 software was used for primer design, with an amplicon size set to 100 – 200 bp. The characteristics of the key genes and their primers are described in Table 1.

Each qRT–PCR reaction was performed in a 15 µl containing 1X Maxima SYBR Green/ROX qPCR master reagent (Thermo Scientific, USA), 0.3 µM of each primer, and 1 µl of first-strand cDNA mixture. Control reactions lacking DNA templates were included. The qRT–PCR cycling conditions were: initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 20 seconds. Fluorescence readings were recorded at 72°C, and melting curve analysis was conducted using a StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA). Agarose gel electrophoresis confirmed the PCR product sizes, matching each gene's expected sizes. Target gene identity was reconfirmed by Sanger sequencing and BLASTn homology searches against GenBank.

All samples were processed in triplicate along with serial dilutions of plasmid DNA containing the target sequence to create a standard curve for gene expression analysis. The standard curve determined the real-time PCR baseline and assay efficiency ($E = 10^{-(1/slope)} - 1$) as described by Svec et al. (2015), ranging from 91.3% to 99.0%. The reproducibility (R^2) was always > 0.99. Basal mRNA transcripts of *cyp19a* and *cyp19b* were normalized to *18S rRNA* from the same samples and calculated using the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008). The fold change in expression relative to unpaired fish at the same age was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), as described by Boonphakdee et al. (2019).

2.5. Statistical analysis

A one-way ANOVA was performed using the SPSS software package, and post hoc comparisons were conducted using Duncan's test at a 95% confidence level ($P<0.05$) to analyze the specific growth rate.

Table 1. Characteristics of the key genes and their primers used for the qRT–PCR in false clown anemonefish.

Gene	Primer sequences (5´-3´) forward/reverse	Amplicon length (bp)	Melting Tm (°C)	Efficiency (%)	Correlation coefficient (R^2)
cyp19a1a	CGATGGGGAGGAGACACTAA/ CGGATCTTCTTCCACAGAGC	168	79.9	91.3	0.99
cyp19a1b	GAACATGGTACACCGCAGAC/ GCCCGATTTTCTCCTACACA	150	81.4	104.2	0.99
18S rRNA	CGAGGAATTCCCAGTAAG/ CTCACTAAACCATCCAATC	102	80.8	99.0	0.99

For gene expression analysis, the $2^{-\Delta\Delta CT}$ method was used to measure the expression levels of the target and reference genes by analyzing the Ct values after completing the qRT-PCR reaction. One-way ANOVA with Duncan's test at a 95% confidence level was used to compare the differences between the test groups.

3. Results

3.1. Growth in a false clown anemonefish

In this study, we monitored the specific growth rates (SGR % day^{-1}) of the subadult false clown anemonefish following pairing for different durations (0, 1, 2, 3, 4, 5, and 16 months). We found that at 2 months of pairing (10 months old), the paired females showed the highest SGR (0.048 ± 0.007% day^{-1}), while paired males and unpaired controls exhibited the lowest SGR (0.018 ± 0.005% day^{-1} and 0.011 ± 0.004% day^{-1}, respectively) (see Figure 2). Additionally, significant differences were observed in size (total length; L_t data not shown) between the couples starting from this age (paired males = 50.75 ± 0.83 mm, paired females = 53.40 ± 0.79 mm). However, the SGR continued to decrease beyond this time. At 16 months of pairing, the highest difference between paired males and paired females was observed, with paired females having a significantly higher total length (66.60 ± 1.67 mm) compared to paired males (56.41 ± 2.72 mm) (see Figure 2). Therefore, the differences between paired males and females may be related to the pairing event, as no significant differences were observed in the unpaired controls.

3.2. Gonadal development in false clown anemonefish

This study investigated gonadal development in false clown anemonefish after pairing. Male fish showed decreased ovarian tissue and a significant increase in testicular tissue upon pairing, with spermatozoa becoming more abundant until they became complete males at 16 months (see Figure 3K, 3L, 3M, and 3N). In contrast, cortical alveolar oocytes appeared only in paired females, beginning at 3 months of pairing and increasing in number and size over time (see Figure 3R, 3S, 3T, and 3U). Quantitative data showed that paired females exhibited increased ovarian tissue, ranging from 82.00 ± 0.85% to 100.00 ± 4.29%, while paired males had a marked increase in testicular tissue, from 17.00 ± 1.01% to 96.00 ± 0.64%. At specific time points, paired females exhibited a higher percentage of ovarian tissue than unpaired controls, while paired males had a decrease in ovarian tissue over time (see Figures 3 and 4). Our study identified a distinct subgroup of paired anemonefish that displayed advanced gonadal development, resembling the paired mature adults after 16 months of pairing. To complete the sex change to female, paired females require a minimum of 5 months of pairing, as observed in this subgroup at 13 months old. The false clown anemonefish undergo a gradual sex change process, with paired females shifting toward female gonadal development and completely losing the testicular tissue (see Figure 3U). In contrast, paired males experience decreased ovarian tissue, with 4% ovarian region in paired males at 24 months old (see Figures 3 and 4).

3.3. Expression levels and sex changes in the false clown anemonefish

Our study examined the relationship between *cyp19a1a* and *cyp19a1b* expression levels and sex changes in false clown anemonefish. After one month of pairing, we found that males and females had slightly higher *cyp19a1a* expression levels than unpaired controls. At 2 months of pairing, *cyp19a1a* expression levels remained similar in paired males (1.13 ± 0.12) but increased slightly in paired females (1.46 ± 0.13). At three months of pairing, *cyp19a1a* expression levels significantly increased in both sexes, with females having higher levels than males. *Cyp19a1a* expression levels continued to increase in females up to 5 months of pairing, while they decreased in males. At the end of the experiment, sexually mature females at 24 months old had the highest expression of *cyp19a1a* at 16.93 ± 2.53, while unpaired controls had slightly higher levels. In contrast, the partner males had the lowest expression of *cyp19a1a* throughout the experiment (see Figure 5A).

Regarding *cyp19a1b* expression levels, we found that the expression levels of *cyp19a1b* appeared to increase with age and pairing time, with noticeable differences between paired males and females beginning at 2 months of pairing with an expression value of 1.76 ± 0.49 for paired males and 2.07 ± 0.14 for paired females.

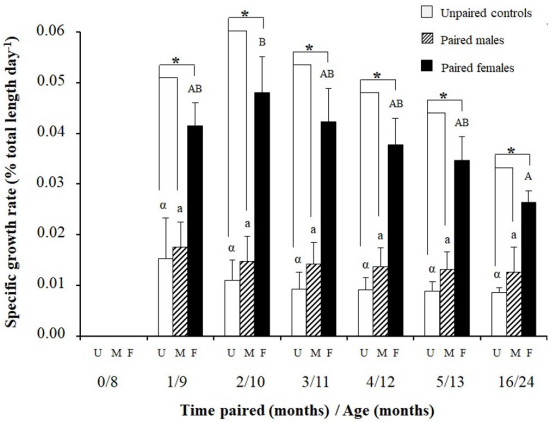

Figure 2. Growth comparison of the paired and unpaired false clown anemonefish (*Amphiprion ocellaris*). Specific growth rates (SGR % day^{-1}) were compared between experimental fish paired for 0, 1, 2, 3, 4, 5, and 16 months (n = 4; each age and gender group; triplicate assays). SGR was calculated using total length values per rearing day following Lugert et al. (2014). Data are presented as means ± S.E.M. No significant difference is indicated within unpaired controls (U = unpaired controls; α) and paired males (M = paired males; a). Different letters indicate a significant difference within paired females (F = paired females; A, B). Asterisks (*) denote significant differences within the same age group. Data were analyzed using one-way ANOVA and Duncan's 95% confidence-level test.

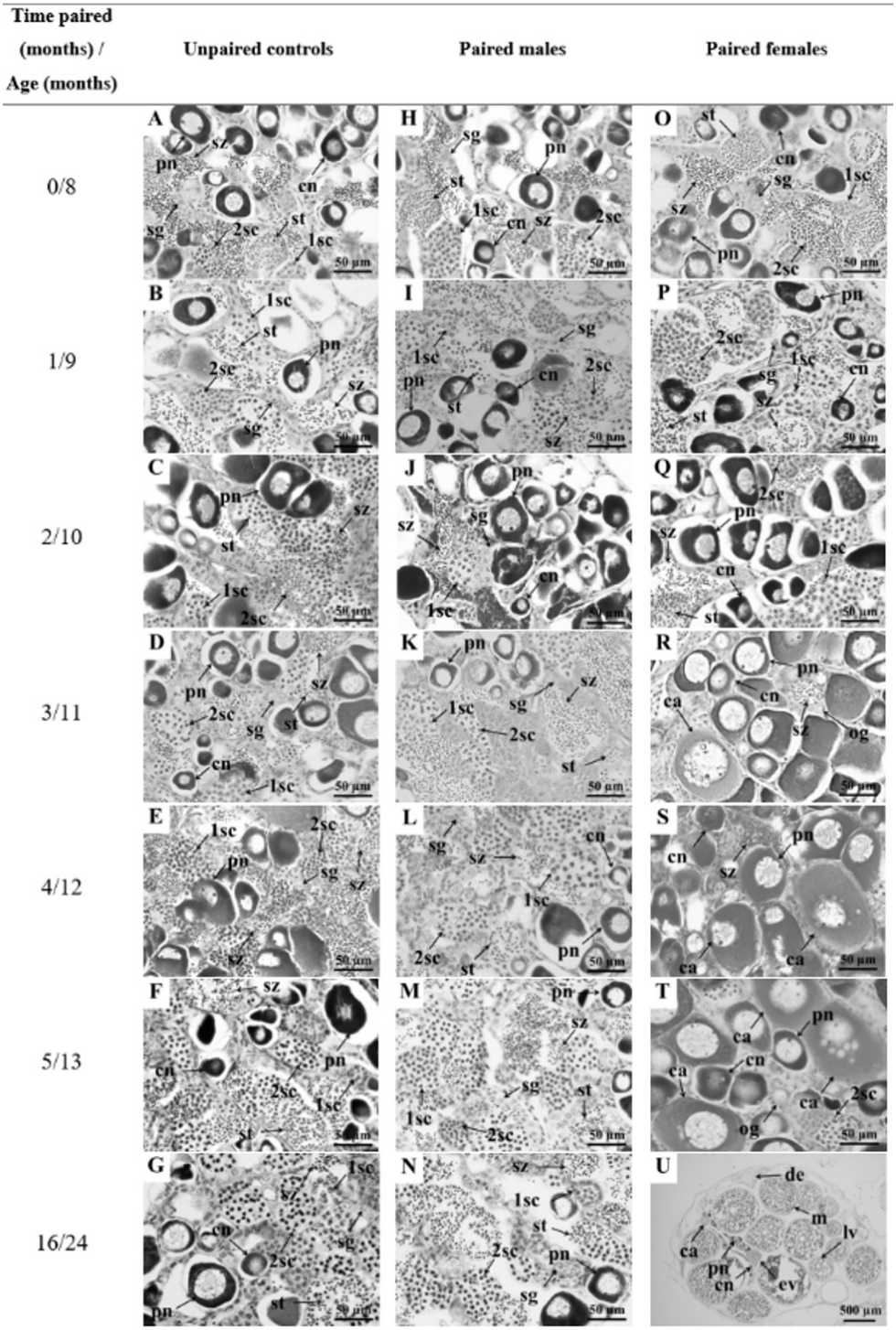

Figure 3. Gonadal development in paired and unpaired false clown anemonefish (*Amphiprion ocellaris*) subadults. Histological sections of gonadal tissue from subadults aged 8-13 and 24 months at different post-pairing times (0, 1, 2, 3, 4, 5, and 16 months) show gonadal development progression and differences between paired and unpaired fish. Gonads from unpaired controls (A-G), paired males (H-N), and paired females (O-U) were examined (n = 4 per age and gender, 3 slides per sample). Testicular stages: spermatogonia (sg), primary spermatocyte (1sc), secondary spermatocyte (2sc), spermatids (st), spermatozoa (sz), ovarian stages: oogonia (og), chromatin nucleolus (cn), perinucleolar oocytes (pn), cortical alveolar oocytes (ca), early vitellogenic oocytes (ev), late vitellogenic oocytes (lv), and mature oocytes (m). At 16 months, paired males (T) showed reduced ovarian tissue, primary nucleolus (pn), and all testicular stages, while paired females (U) displayed all ovarian stages and degenerated testicular tissue (de). Sections were stained with hematoxylin and eosin. Scale bar = 50 µm (A-T) and 500 µm (U).

Significant differences were found at the age of 13 months and beyond. Furthermore, the expression levels of *cyp19a1b* were significantly higher in paired females than in unpaired controls at all examined time points, with the highest expression level observed at 16 months of pairing. The expression levels of *cyp19a1b* in paired males were also higher than those in unpaired controls, but the difference was not statistically significant (see Figure 5B).

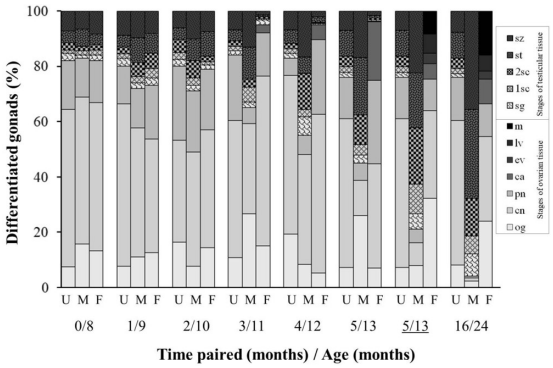

Figure 4. Gonadal cell composition in the paired and unpaired false clown anemonefish (*Amphiprion ocellaris*) subadults. The diagram shows the percentage of various gonadal cell types in unpaired controls (U), paired males (M), and paired females (F) at different post-pairing times (0, 1, 2, 3, 4, 5, and 16 months) for subadults aged 8-13 and 24 months. Testicular cell types: spermatogonia (sg), primary spermatocyte (1sc), secondary spermatocyte (2sc), spermatids (st), spermatozoa (sz), ovarian cell types: oogonia (og), chromatin nucleolus (cn), perinucleolar oocytes (pn), cortical alveolar oocytes (ca), early vitellogenic oocytes (ev), late vitellogenic oocytes (lv), and mature oocytes (m). Data presented as mean with standard error (S.E.M), n = 3 per age, and gender. A distinct subgroup was observed in 5-month paired individuals (5/13) with characteristics similar to paired mature adults.

Notably, the unique 5/13 subgroup (see Figure 5B; 5/13) showed significantly higher *cyp19a1b* expression levels in males and females than in other paired fish. These results suggest that *cyp19a1b* expression levels are age- and sex-dependent, and that the 5/13 subgroup may represent a faster-developing anemonefish with potentially different reproductive strategies.

4. Discussions

Our study provides novel insights into the growth patterns, gonadal development, and sex change processes in the false clown anemonefish, *A. ocellaris*. Our observations suggest that social interactions and pairing events play a pivotal role in the sex change process, as females exhibited a higher specific growth rate (SGR) than males during the initial two months of pairing, potentially facilitating the process of sex change. Our study also revealed a trade-off between growth and gonadal development during sex change, which is consistent with previous research on various fish species (Hattori, 2000; Abol-Munafi et al., 2011; Madhu et al., 2012; Lugert et al., 2014). Our findings suggest that gonadal development in the false clown anemonefish is influenced by pairing duration and social cues, with paired females exhibiting increased ovarian tissue, and paired males showing a marked increase in testicular tissue (Madhu et al., 2012). The observed differences in gonadal development between paired and unpaired fish underscore the significance of pairing in the sex change process (Moyer and Nakazono, 2016). The observed differences in gonadal development between paired and unpaired fish highlight the significance of pairing in the sex change process (Moyer and Nakazono, 2016). Including a control group in separate tanks with unpaired fish is important to understand the changes observed in paired fish.

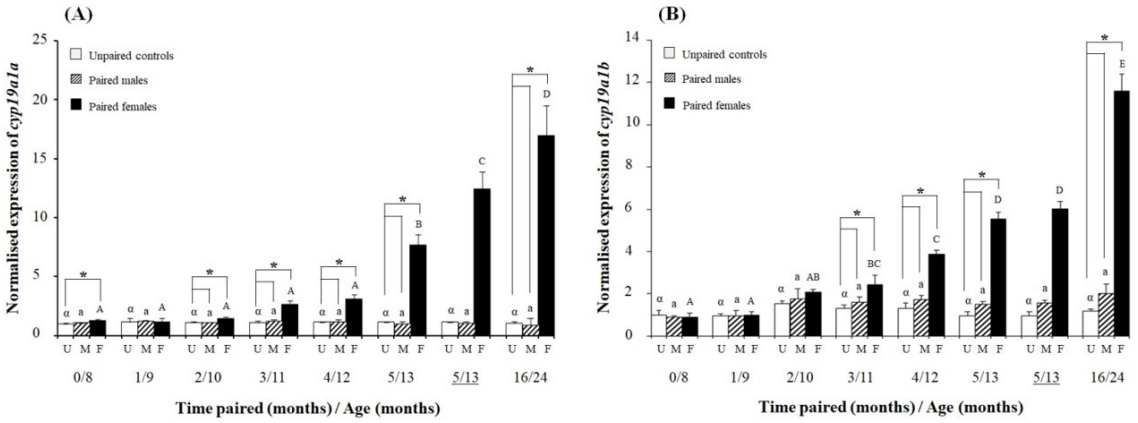

Figure 5. Expression levels of *cyp19a1a* and *cyp19a1b* in paired and unpaired subadults of the false clown anemonefish at different ages and durations. The expression levels of *cyp19a1a* mRNA in the gonadal tissue (A) and *cyp19a1b* mRNA in the brain (B) were determined using qRT-PCR. The relative quantity of *cyp19a1a* and *cyp19a1b* mRNA transcripts was normalized to the expression of *18S rRNA*. The data are shown as mean ± S.E.M. and fold changes relative to unpaired controls at the initial time point (n = 4 per sample per gender, each with triplicate qRT–PCR). Asterisks (*) indicate differences within the group ($P<0.05$). No significant differences were observed within unpaired controls and paired males, indicated by the same letter (α and a, respectively) ($P\geq0.05$). Uppercase letters (A, B, C, D, and E) indicate differences within paired females. One-way ANOVA followed by Duncan's post-hoc test determined statistical significance. A subgroup 13-month pair (5/13) exhibited high gene expression levels in paired females (U = unpaired controls, M = paired males, and F = paired females).

By comparing the expression levels of *cyp19a1a* and *cyp19a1b* in the control group to those observed in paired females and males and through histological examination of gonadal tissues, we can identify the changes specifically associated with the pairing process. The relatively constant *cyp19a1a* and *cyp19a1b* expression levels in unpaired controls suggest that these changes are not simply due to age-related differences or other factors, but are related to the social cues and interactions involved in the pairing process. These findings emphasize the importance of using appropriate controls in experimental designs to ensure accurate interpretation of the data.

We identified *cyp19a1a* and *cyp19a1b* as genes associated with sex change in false clown anemonefish. As discussed in the review by Rajendiran et al. (2021), the *cyp19a1a* gene is crucial for encoding the aromatase enzyme, which is responsible for converting androgens to estrogens, playing a significant role in sexual development and reproduction in teleost fish. Our results indicate that *cyp19a1a* expression levels increased in paired females, whereas they decreased in paired males, suggesting a role in *cyp19a1a* in sex change regulation (Ijiri et al., 2008). These findings align with Casas et al. (2016), who also identified *cyp19a1a* as a gene associated with sex change in clownfish. Their transcriptome analysis revealed increased *cyp19a1a* expression in the gonads of transitioning males. Additionally, Kobayashi et al. (2010) reported that *cyp19a1a* is restricted to the ovarian follicle of female *A. clarkii*, indicating that aromatase may play a crucial role in sex change in this species. Regarding *cyp19a1b*, our study found that expression levels increased with age and pairing time and were higher in paired females than in unpaired controls. The elevated expression levels of *cyp19a1b* in the unique 5/13 subgroup indicate a potential link to faster-developing anemonefish with potentially different reproductive strategies (Forlano et al., 2001; Kishida and Callard, 2001).

In addition to our findings on gonadal development and gene expression, we observed that the male-to-female sex change process in *A. ocellaris* appears irreversible, consistent with previous studies on protandrous hermaphroditic fish species (Godwin, 2010). This irreversible sex change may be due to differences in gene expression and hormonal signaling during the transition, suppressing male reproductive development and promoting female development (Hattori, 2000; Liu et al., 2015a).

Our findings on false clown anemonefish sex change have similarities and differences compared to other fish species exhibiting sex changes, such as *Thalassoma bifasciatum* (bluehead wrasse), *Danio rerio* (zebrafish), *Lythrypnus dalli* (blue-banded goby), *Labroides dimidiatus* (cleaner wrasse), and *Anguilla japonica* (Japanese eel). The trade-off between growth and gonadal development during sex change is a common theme across these species, but the specific factors and mechanisms driving this trade-off may differ (Ijiri et al., 2008; Kobayashi et al., 2013). For instance, in the protogynous bluehead wrasse, sex change involves rapid gonadal development, accompanied by decreased body growth (Thomas et al., 2019). In zebrafish, estrogen treatment promotes female development at the expense of body growth (Liu et al., 2015b). The blue-banded goby and cleaner wrasse have different mechanisms regulating sex change, with the former undergoing a rapid increase in both body and gonadal development and the latter experiencing rapid growth and gonadal development (Munday et al., 2006; Godwin, 2010; Warner et al., 2015). In Japanese eel, increased *cyp19a1a* and *cyp19a1b* expression is associated with the development of ovarian tissue, while reduced expression is associated with the development of testicular tissue (Ijiri et al., 2008). Finally, our study highlights the importance of the *cyp19* genes in the sex change process in *A. ocellaris*, with *cyp19a1a* and *cyp19a1b* expression levels correlating with gonadal development and sex change. The expression patterns observed in our study align with previous findings in other fish species, suggesting that the regulation of these genes may be conserved across different taxa. However, more research is needed to determine the specific mechanisms underlying the regulation of *cyp19a1a* and *cyp19a1b* in sex change in *A. ocellaris*.

Based on our observations, future research could investigate the factors that influence the rate and timing of sex change in *A. ocellaris*, including the effects of size differences between individuals, growth rates, feeding patterns, and aggressive behaviors. The expression of *cyp19* genes, which encode the aromatase enzyme responsible for the conversion of androgens to estrogens, can be influenced by various factors in both the gonads and the brain. The regulation of *cyp19* gene expression involves a combination of external and internal factors. Hormonal regulation plays a crucial role in controlling *cyp19* gene expression. In the gonads, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have been shown to stimulate *cyp19* gene expression and aromatase activity (Simpson et al., 1997; Enjuanes et al., 2003). Similarly, in the brain, various hormones, including gonadotropin-releasing hormone (GnRH), estradiol, and progesterone, can modulate the expression of *cyp19* genes (Simpson et al., 1997; Enjuanes et al., 2003).

Transcription factors also play a significant role in regulating *cyp19* gene expression. For example, the transcription factor steroidogenic factor 1 (SF-1) has been identified as an important regulator of *cyp19* gene expression in the brain (Simpson et al., 1997). Additionally, gonadal development itself influences the expression of *cyp19* genes. In females, *cyp19* gene expression is typically higher in the ovaries than in males, and this sexual dimorphism is regulated by sex-determining genes and downstream factors involved in gonadal differentiation (Zhao et al., 1997).

While considerable research has focused on hormonal regulation and gonadal development, investigations into other factors such as body size, food intake, and dominance in relation to *cyp19* gene expression are limited in fish. The influence of these factors and other internal factors, including the expression of transcription factors and hormones, on the regulation of *cyp19* gene expression in both the gonads and the brain remains an area that requires further investigation.

Note that sex change is a natural process in the false clownfish and may take up to 5 months to complete, especially when starting with fish of similar sizes.

Therefore, it is important to strike a balance between optimizing the efficiency of sex change and ensuring the health and well-being of the fish. Measuring serum levels of 17β-estradiol and testosterone at different time points during the sex change process could provide further evidence for involving *cyp19a1a* and *cyp19a1b* in sex change regulation in *A. ocellaris*. Decreased serum 17β-estradiol levels in paired males and increased levels in paired females may indicate the involvement of *cyp19a1a* in the sex change process (Nakamura et al., 2015). On the other hand, increased testosterone levels in paired males and decreased levels in paired females may suggest the involvement of *cyp19a1b*. However, further studies are necessary to confirm these hypotheses.

Our observations have revealed that when pairing false clown anemonefish, there is a higher incidence of aggression from paired females towards males. This finding suggests the presence of social dynamics during the pairing process. Based on this observation, we propose a hypothesis that reducing aggression levels during pairing while also considering the size difference between males and females (with a minimum difference of 2.5 mm, as observed two months after pairing) could potentially lead to faster sex changes and increased breeding success in false clown anemonefish.

Further research is warranted to validate this hypothesis and better understand the underlying mechanisms. Specifically, investigating the influence of aggression levels and size differences on sex change rates and breeding success in false clownfish would be valuable. By further understanding these factors, it may be possible to optimize the management practices of captive breeding programs for anemonefish species, ultimately improving their sustainability in captivity. Additionally, our study highlights the importance of the *cyp19* genes in the sex change process in *A. ocellaris*, with *cyp19a1a* and *cyp19a1b* expression levels correlating with gonadal development and sex change. The expression patterns observed in our study align with previous findings in other fish species, suggesting that the regulation of these genes may be conserved across different taxa. However, more research is needed to determine the specific mechanisms underlying the regulation of *cyp19a1a* and *cyp19a1b* in sex change in *A. ocellaris*.

5. Conclusion

In conclusion, our study investigated the growth, gonadal development, and expression of *cyp19a1a* and *cyp19a1b* in false clown anemonefish. Our findings indicate that paired females had higher specific growth rates than paired males. We also observed a gradual sex change process in the false clown anemonefish, with paired females shifting toward female gonadal development and paired males experiencing increased testicular tissue. The onset of sex change initiation occurs after 3 months of pairing, with the appearance of cortical alveolar oocytes. The complete transformation to female requires a minimum of 5 months of pairing. Our study provides valuable insights into the growth and sex change processes in false clown anemonefish and highlights the importance of social cues such as pairing in regulating sex change.

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References

ABOL-MUNAFI, A.B., NADIRAH, M. and YUSOFF, F., 2011. Sex ratio and morphometric relationship of clownfish *Amphiprion sebae* in the wild and captivity. *Fisheries and Aquatic Sciences*, vol. 6, pp. 75-83.

BOONPHAKDEE, C., OCHAROEN, Y., SHINN, A.P., SUANLA, S. and THAMNAWASOLOS, J., 2019. *18S rRNA*, a potential reference gene in the qRT-PCR measurement of bisphenol A contamination in green mussels (*Perna viridis*) collected from the Gulf of Thailand. *Agriculture and Natural Resources*, vol. 53, no. 6, pp. 652-661. http://dx.doi.org/10.34044/j.anres.2019.53.6.13.

BUSTON, P., 2003. Size and growth modification in clownfish. *Nature*, vol. 424, no. 6945, pp. 145-146. http://dx.doi.org/10.1038/424145a. PMid:12853944.

CASAS, L., SABORIDO-REY, F., RYU, T., MICHELL, C.T., RAVASI, T. and IRIGOIEN, X., 2016. Sex change in clownfish: molecular insights from transcriptome analysis. *Scientific Reports*, vol. 6, no. 1, p. 35461. http://dx.doi.org/10.1038/srep35461. PMid:27748421.

DEVLIN, R.H. and NAGAHAMA, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, vol. 208, no. 3-4, pp. 191-364. http://dx.doi.org/10.1016/S0044-8486(02)00057-1.

DIETRICH, D.R. and KRIEGER, H.O., 2009. *Histological analysis of endocrine disruptive effects in small laboratory fish*. Hoboken: John Wiley & Sons, 341 p. http://dx.doi.org/10.1002/9780470431795.

ENJUANES, A., GARCIA-GIRALT, N., SUPERVIA, A., NOGUÉS, X., MELLIBOVSKY, L., CARBONELL, J., GRINBERG, D., BALCELLS, S. and DÍEZ-PÉREZ, A., 2003. Regulation of CYP19 gene expression in primary human osteoblasts: effects of vitamin D and other treatments. *European Journal of Endocrinology*, vol. 148, no. 5, pp. 519-526. http://dx.doi.org/10.1530/eje.0.1480519. PMid:12720534.

FORLANO, P.M., DEITCHER, D.L., MYERS, D.A. and BASS, A.H., 2001. Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as the source. *The Journal of Neuroscience*, vol. 21, no. 22, pp. 8943-8955. http://dx.doi.org/10.1523/JNEUROSCI.21-22-08943.2001. PMid:11698605.

FRICKE, H. and FRICKE, S., 1977. Monogamy and sex change by aggressive dominance in coral reef fish. *Nature*, vol. 266, no. 5605, pp. 830-832. http://dx.doi.org/10.1038/266830a0. PMid:865603.

GODWIN, J., 2010. Neuroendocrinology of sexual plasticity in teleost fishes. *Frontiers in Neuroendocrinology*, vol. 31, no. 2, pp. 203-216. http://dx.doi.org/10.1016/j.yfrne.2010.02.002. PMid:20176046.

GUIGUEN, Y., FOSTIER, A., PIFERRER, F. and CHANG, C.F., 2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *General and Comparative Endocrinology*, vol. 165, no. 3, pp. 352-366. http://dx.doi.org/10.1016/j.ygcen.2009.03.002. PMid:19289125.

HATTORI, A., 2000. Competitive mechanisms underlying social hierarchy formation in clown anemonefish. *Journal of Ethology*, vol. 18, pp. 13-19.

IJIRI, S., KAZETO, Y., LOKMAN, P.M., ADACHI, S., YAMAUCHI, K. and TRANT, J.M., 2008. Isolation and characterization of a *cyp19a1a* gene from Japanese eel *Anguilla japonica*. *Molecular and Cellular Endocrinology*, vol. 291, no. 1-2, pp. 130-138.

KISHIDA, M. and CALLARD, G.V., 2001. Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development. *Endocrinology*, vol. 142, no. 2, pp. 740-750. http://dx.doi.org/10.1210/endo.142.2.7928. PMid:11159846.

KOBAYASHI, Y., HORIGUCHI, R., MIURA, S. and NAKAMURA, M., 2010. Sex- and tissue-specific expression of P450 aromatase (*cyp19a1a*) in the yellowtail clownfish, *Amphiprion clarkii*. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, vol. 155, no. 2, pp. 237-244. http://dx.doi.org/10.1016/j.cbpa.2009.11.004. PMid:19913632.

KOBAYASHI, Y., NAGAHAMA, Y. and NAKAMURA, M., 2013. Diversity and plasticity of sex determination and differentiation in fishes. *Sexual Development*, vol. 7, no. 1-3, pp. 115-125. http://dx.doi.org/10.1159/000342009. PMid:22948719.

LIU, H., LAMM, M.S., RUTHERFORD, K., BLACK, M.A., GODWIN, J.R. and GEMMELL, N.J., 2015a. Large-scale transcriptome sequencing reveals novel expression patterns for key sex-related genes in a sex-changing fish. *Biology of Sex Differences*, vol. 6, no. 1, p. 26. http://dx.doi.org/10.1186/s13293-015-0044-8. PMid:26613014.

LIU, Q., LIU, X., TONG, S. and SHI, B., 2015b. Knockdown of *cyp19a* and *cyp19b* genes encoding aromatase in zebrafish larvae affects development of the ovary and the production of female hormones. *The Journal of Endocrinology*, vol. 224, no. 1, pp. 77-88.

LIVAK, K.J. and SCHMITTGEN, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, vol. 25, no. 4, pp. 402-408. http://dx.doi.org/10.1006/meth.2001.1262. PMid:11846609.

LUGERT, V., THALLER, G., TETENS, J., SCHULZ, C. and KRIETER, J., 2016. A review on fish growth calculation: multiple functions in fish production and their specific application. *Reviews in Aquaculture*, vol. 8, no. 1, pp. 30-42. http://dx.doi.org/10.1111/raq.12071.

LUGERT, V., THALLER, G., TETENS, J., SCHULZ, C. and KRIETER, J., 2014. Genetic associations between growth and reproduction in different age classes of turbot (*Scophthalmus maximus*). *Journal of Animal Science*, vol. 92, no. 5, pp. 1945-1954.

MADHU, K., MADHU, R. and ANILKUMAR, G., 2012. Length–weight relationship and condition factor of two ornamental fish species (Pomacentridae: *Amphiprion ocellaris* and Pomacanthidae: *Holacanthus ciliaris*) of the family Pomacentridae and Pomacanthidae of Lakshadweep islands, India. *Indian Journal of Geo-Marine Sciences*, vol. 41, no. 5, pp. 421-427.

MOYER, J.T. and NAKAZONO, A., 2016. The presence of a dominant female facilitates female-to-male sex change in the clown anemonefish *Amphiprion percula*. *Journal of Fish Biology*, vol. 89, no. 6, pp. 2949-2959.

MUNDAY, P.L., BUSTON, P.M. and WARNER, R.R., 2006. Diversity and flexibility of sex-change strategies in animals. *Trends in Ecology & Evolution*, vol. 21, no. 2, pp. 89-95. http://dx.doi.org/10.1016/j.tree.2005.10.020. PMid:16701480.

NAKAMURA, M., MIURA, S., NOZU, R. and KOBAYASHI, Y., 2015. Opposite-directional sex change in functional female protandrous anemonefish, *Amphiprion clarkii*: effect of aromatase inhibitor on the ovarian tissue. *Zoological Letters*, vol. 1, no. 1, p. 30. http://dx.doi.org/10.1186/s40851-015-0027-y. PMid:26605075.

PARENTI, L.R. and GRIER, H.J., 2004. Evolution and phylogeny of gonad morphology in bony fishes. *Integrative and Comparative Biology*, vol. 44, no. 5, pp. 333-348. http://dx.doi.org/10.1093/icb/44.5.333. PMid:21676719.

RAJENDIRAN, P., JAAFAR, F., KAR, S., SUDHAKUMARI, C., SENTHILKUMARAN, B. and PARHAR, I.S., 2021. Sex determination and differentiation in teleost: roles of genetics, environment, and brain. *Biology*, vol. 10, no. 10, p. 973. http://dx.doi.org/10.3390/biology10100973. PMid:34681072.

SCHMITTGEN, T.D. and LIVAK, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*, vol. 3, no. 6, pp. 1101-1108. http://dx.doi.org/10.1038/nprot.2008.73. PMid:18546601.

SIMPSON, E.R., MICHAEL, M.D., AGARWAL, V.R., HINSHELWOOD, M.M., BULUN, S.E. and ZHAO, Y., 1997. Expression of the CYP19 (aromatase) gene: an unusual case of alternative promoter usage. *The FASEB Journal*, vol. 11, no. 1, pp. 29-36. http://dx.doi.org/10.1096/fasebj.11.1.9034163. PMid:9034163.

SVEC, D., TICHOPAD, A., NOVOSADOVA, V., PFAFFL, M.W. and KUBISTA, M., 2015. How good is a PCR efficiency estimate: recommendations for precise and robust qPCR efficiency assessments. *Biomolecular Detection and Quantification*, vol. 3, pp. 9-16. http://dx.doi.org/10.1016/j.bdq.2015.01.005. PMid:27077029.

THOMAS, J.T., TODD, E.V., MUNCASTER, S., LOKMAN, P.M., DAMSTEEGT, E.L., LIU, H., SOYANO, K., GLÉONNEC, F., LAMM, M.S., GODWIN, J.R. and GEMMELL, N.J., 2019. Conservation and diversity in expression of candidate genes regulating socially-induced female-male sex change in wrasses. *PeerJ*, vol. 7, p. e7032. http://dx.doi.org/10.7717/peerj.7032. PMid:31218121.

WARNER, R.R., SWEARER, S.E. and WU, T., 2015. Social cues trigger seasonal changes in sex phenotype and reproductive physiology in the blue banded goby, *Lythrypnus dalli*. *Journal of Fish Biology*, vol. 86, no. 3, pp. 1021-1035.

ZHAO, Y., AGARWAL, V.R., MENDELSON, C.R. and SIMPSON, E.R., 1997. Transcriptional regulation of *CYP19* gene (aromatase) expression in adipose stromal cells in primary culture. *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 61, no. 3-6, pp. 203-210. http://dx.doi.org/10.1016/S0960-0760(97)80013-1. PMid:9365191.