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ORIGINAL RESEARCH

Exploring the growth patterns, gonadal development, and expression of *igf1* in captive-reared false clown anemonefish (*Amphiprion ocellaris*): Implications for breeding programs and reproductive biology

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Abstract Understanding the relationship between growth and gonadal development is essential for breeding programs and studying teleost sexual development. This study investigated the growth and gonadal development of captive-reared false clown anemonefish (*Amphiprion ocellaris*) aged one to eight months. We performed histological analysis and quantified insulin-like growth factor-1 (*igf1*) mRNA transcripts. The fish exhibited a maximum specific growth rate of 0.56 ± 0.11% day^{-1} at two months, with a negative allometric length-weight relationship. Histological examination revealed ovarian differentiation commenced in the third month, while male germ cells progressed rapidly. Ovotestis formation occurred between the fourth and eighth month, with primary growth stage oocytes being predominant. Ovarian tissue consistently occupies a larger area than testicular tissue. Expression levels of *igf1* in the liver peaked at eight months, with the highest expression observed in gonadal tissues at two months, which decreased significantly in older fish. These findings suggest a potential role *igf1* in ovarian differentiation and the growth of primary oocytes. Further investigations are warranted to explore the interplay between *igf1* expression and other regulatory factors. This research enhances our understanding of fish reproductive biology and has implications for the captive management of false clownfish.

Keywords Captive breeding . False clownfish . Gonadal maturation . Growth regulation . Insulin-like growth factor 1 (IGF-1) . Reproductive physiology

Introduction

The false clown anemonefish (*Amphiprion ocellaris* Cuvier 1830) is renowned for its distinctive coloration and patterning, making it a valuable and highly desirable ornamental species, as well as serving as a model organism for studying growth regulation and reproductive development in teleosts (Wabnitz et al. 2003; Rhyne et al. 2012). Despite its significance in these research areas and its popularity in the aquarium trade, there are still substantial gaps in our understanding of the relationship between growth and gonadal differentiation during the early developmental months of this species. A comprehensive understanding of this domain is crucial for optimizing breeding programs and enhancing our knowledge of sexual development in teleost fish (Buston 2003).

In the case of breeding ornamental fish like the false clown anemonefish, captive rearing provides the advantage of controlled environmental conditions and optimal nutrition, leading to increased survival rates

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and accelerated growth (Olivotto et al. 2011). It is important to note, however, that the growth, development, and reproduction of these fish are influenced by various factors, including environmental conditions (such as diet, temperature, photoperiod, chemicals, and oxygen), social cues, and genetic variations (Godwin 2009; Gjedrem et al. 2012; Liu et al. 2022).

One notable characteristic of false clown anemonefish is the formation of breeding pairs of larger females and smaller males (Fautin and Allen 1992; Buston 2003; Godwin 2009). Sub-adults and juveniles possess ambisexual gonads and do not exhibit sexual functionality until the later stages (Fautin and Allen 1992; Buston 2003; Godwin 2009). Genetic factors, environmental cues, and social interactions influence the gonadal development and differentiation process of these fish (Casas et al. 2016). In a study conducted by Madhu et al. (2012), the life cycle of false clownfish was observed to encompass various stages: embryonic, larval, juvenile, sub-adult, adult, and senescence. Rapid growth occurs during the juvenile stage, with juveniles reaching a size of 40-60 mm within 3-6 months. The juvenile stage persists until the first maturation of gametes, and sub-adults experience further growth, achieving reproductive maturity at around 18 months. This extended time for reaching the adult stage poses limitations for enhancing captive breeding in aquaculture.

The hormone-insulin-like growth factor-1 (IGF-1), primarily synthesized in the liver and other tissues, plays a significant role in the regulation of growth and reproductive development across vertebrates (Duan et al. 2010; Reinecke 2010a; Casas et al. 2016). Its production and function are stimulated by growth hormone (GH), secreted predominantly by the pituitary gland, and encoded by the *gh* gene (Vázquez-Borrego et al. 2021). This hormone duo of GH and IGF-1 boosts somatic growth, skeletal development, and teleost reproduction (Reinecke 2010a; 2010b) while simultaneously regulating metabolic processes such as lipid metabolism, protein synthesis, and energy balance (Wang et al. 2020; Vázquez-Borrego et al. 2021).

IGF-1 also critically influences gonadal development and sexual maturation in fish by regulating germ cell proliferation, survival, and differentiation within the developing gonads (Xie et al. 2022). It impacts the differentiation of undifferentiated germ cells into oocytes or spermatozoa during the early stages of gonadal development (D'Ercole 1996). Additionally, IGF-1 promotes the growth and maturation of gonads in juvenile fish, fostering the development and maturation of gametes. Its expression is regulated by various hormonal signals, including GH and sex steroids (such as estrogen and testosterone) (Wood et al. 2005).

Despite extensive studies on the role of GH in growth regulation and reproductive development, our understanding of the specific role of the *igf1* gene-encoded IGF-1, precisely its mRNA expression in the growth and gonadal development of the false clown anemonefish is still limited (Duan et al. 2010; Reinecke 2010a; Casas et al. 2016).

To fill this gap, our study investigates the expression of *igf1* mRNA transcripts in the liver and gonads. Simultaneously, we observed gonadal development by histological examination in captive-reared false clown anemonefish aged one to eight months before they entered the sex-change process. Understanding the role of *igf1* mRNA transcripts in growth and gonadal development may guide the development of enhanced rearing conditions, nutrition strategies, and hormonal manipulations (Triantaphyllopoulos et al. 2020). These improvements can potentially optimize growth rates and reproductive outcomes in the false clown anemonefish, aiding conservation efforts and commercial breeding programs.

Materials and methods

Pathogen-free juvenile false clownfish (n = 80), weighing approximately 0.1 g at three weeks old, were obtained from the Samutsakhon Coastal Fisheries Research and Development Center, Thailand. The fish were transported to the hatchery facility at Burapha University, Chonburi, Thailand. Upon arrival, the fish underwent a seven-day quarantine period in a 500-L seawater tank. The quarantine tank operated as a recirculation system, with appropriate aeration (i.e., >5 mgL^{-1}), and maintained optimal water quality. Daily monitoring and adjustments were made to temperature, salinity, ammonia, nitrite levels, and alkalinity. A 15% daily water exchange was performed using biologically filtered seawater.

Following the quarantine phase, fish of similar sizes (0.18 ± 0.02 g) were evenly distributed among sixteen 120-L tanks (40 cm × 45 cm × 65 cm) containing 90 L of seawater. Four fish were randomly placed in each tank. To ensure adequate spacing and minimize interference, circular plastic netting with a 1 mm mesh diameter was employed for the first two months, maintaining a minimum distance of 5 cm between

individual fish. At three and five months, the netting was replaced with 3 mm and 7 mm mesh diameters, respectively, and this arrangement was maintained until the fish reached eight months of age.

Throughout the experiment period, the fish aged between one and eight months were fed a commercial diet (NRD G8, INVE Aquaculture, Thailand) twice daily at a rate of 15% of their body weight per day. Each week, the seawater in the culture tanks was refreshed by adding new stock seawater that had undergone biological filtration, constituting 20% of the total tank volume. Seawater conditions were monitored regularly to ensure the temperature, salinity, ammonia, nitrite levels, alkalinity, and dissolved oxygen were within the optimal range for the species. The temperature was controlled at 29 ± 1 ºC, salinity was maintained at 30 ppt, and ammonia and nitrite levels were kept below 0.01 mgL^{-1}. Alkalinity was maintained at 120 mgL^{-1}, and dissolved oxygen levels were maintained at 5.19 ± 0.13 mgL^{-1}. For growth rate measurements and analysis, eight fish were randomly sampled from two tanks every month, starting at one month old and concluding at eight months old.

Fish growth measurement

To study the growth of the experimental false clown anemonefish, they were first euthanized using 200 mgL^{-1} MS-222 (Sigma-Aldrich, USA). Their total length (mm) was measured using digital Vernier calipers (Fuzhou Guanghui, China), and body weight (g) was recorded (Lugert et al. 2016). This data was used to analyze the specific growth rate (SGR) and the length-weight relationship (LWR).

The SGR was determined by comparing the differences in the natural logarithm of the fish's initial and final measurements, divided by the number of days between samplings (Lugert et al. 2016). This value was then multiplied by 100 to obtain the growth rate to give a daily percentage. The relationship between body length and weight was analyzed using a logarithmic transformation of the length-weight equation, following that provided by Ricker (1973). In this context, variables, intercept, and slope represented the sample's body weight and total length.

To understand the type of growth the fish exhibited, a comparison was made between the average slope value and a value of 3 (Silva et al. 2015). A Student's t-test with a 5% significance level was applied to determine if the deviation was significant. If the fish displayed isometric growth, the slope value would equal 3. If the fish exhibited negative allometric growth, the slope value would be less than 3, and if it showed positive allometric growth, the slope value would be greater than 3 (Silva et al. 2015).

A one-way analysis of variance (ANOVA) using IBM SPSS Statistics 24 software was performed to assess the significance of the SGR, with *post hoc* multiple comparisons conducted using Duncan's test. Differences with $P < 0.05$ were considered significant for growth rate and condition factors. The data are presented as the mean ± standard deviation (SD).

Histological analysis of gonadal development

A precise approach was used to follow the histological progression of gonadal development. Three out of the eight fish randomly sampled each month were fixed in Davidson's fixative solution for 24 hours. These specimens were then subjected to histological procedures to examine their gonadal development. For fish aged one to three months old, the entire trunk, along with all organs, was fixed *in situ*. In contrast, for fish aged four months and older, the gonads were carefully dissected out and subsequently fixed. The selected tissue samples underwent a series of standard histological procedures. This included the preparation of tissue sections with a thickness of 5 µm, followed by staining using hematoxylin and eosin. Serial longitudinal histological sections were taken through the gonads. Specifically, three consecutive sections were captured from the central region of the gonads for analysis on each slide, as indicated in Fig. 1. To ensure the reliability of the observations, multiple slides and tissues sections from each fish were assessed, i.e., three separate slides and a total of nine sections were carefully examined for each individual fish. Light microscopy, utilizing an Olympus model CX31, facilitated the examination of gonadal developments. Measurements were taken for female and male germ cell areas, as well as gonadal tissue areas, to determine the respective female (or male) gonadal surface area ratio in relation to the overall gonadal area (as depicted in Fig. 1). The assessment of gonadal and germ cell surface areas was conducted using the cellSens software (DP22, Olympus, Japan). Notably, each tissue section underwent a rigorous scoring process and was evaluated in triplicate.

Fish gonadal tissue was systematically classified into three distinct categories based on morphological features, following the criteria outlined by Madhu et al. (2012). These categories comprised the undifferentiated gonad phase (UP), characterized by the prevalence of primordial germ cells (PGCs); the ovarian differentiated phase (OP), marked by ovarian cysts containing primary-growth stage oocytes as the predominant germ cell type; and the testis differentiated phase (TP), characterized by the presence of ovarian elements, oocytes at the cortical-alveolus stage, and cysts of spermatozoa—the most advanced germ cell type of spermatogenesis.

Quantification of *igf1* mRNA transcripts in liver and gonadal tissues by qRT-PCR

Quantitative real-time PCR (qRT-PCR) was performed to determine the relative expression levels of *igf1* in the liver and gonadal tissues of clownfish aged one to eight months old. In addition, the remaining five fish, unused in the histological study, were processed. A pooled sample of livers from two individuals was used for one-month-old fish; however, for fish aged two to eight months, individual livers were processed.

RNA extraction and cDNA synthesis

Total RNA was extracted separately from the liver and gonads of each clownfish using the RNAzol-RT RNA isolation reagent (Molecular Research Center, USA) according to the manufacturer's protocol. The extracted RNA was quantified spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, USA) and stored at -80 ºC until a DNase treatment was performed. According to the manufacturer's instructions, DNase treatment was conducted on 2 µg of the extracted RNA using 1 U of RNase-free DNase (Thermo Scientific, USA). The quality of the RNA was checked by electrophoresis on a 0.8% agarose gel stained with ethidium bromide (Vivantis, USA). The RNA quality was verified through optical density (OD) absorbance ratios $A_{260/280}$, measured on a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA). RNA with an $A_{260/280}$ ratio of 1.8-2.0 was accepted for downstream processes. Subsequently, cDNA was synthesized using a High-capacity cDNA Reverse Transcriptase Kit (Thermo Fisher Scientific, USA). Briefly, 0.5 µg of DNase-treated RNA was added to an RNase-free 200-µl PCR tube (Axygen Scientific, USA) along with 2 µl of 10X RT Random Primers, 0.8 µl of 25X dNTP Mix (100 mM), 2 µl of

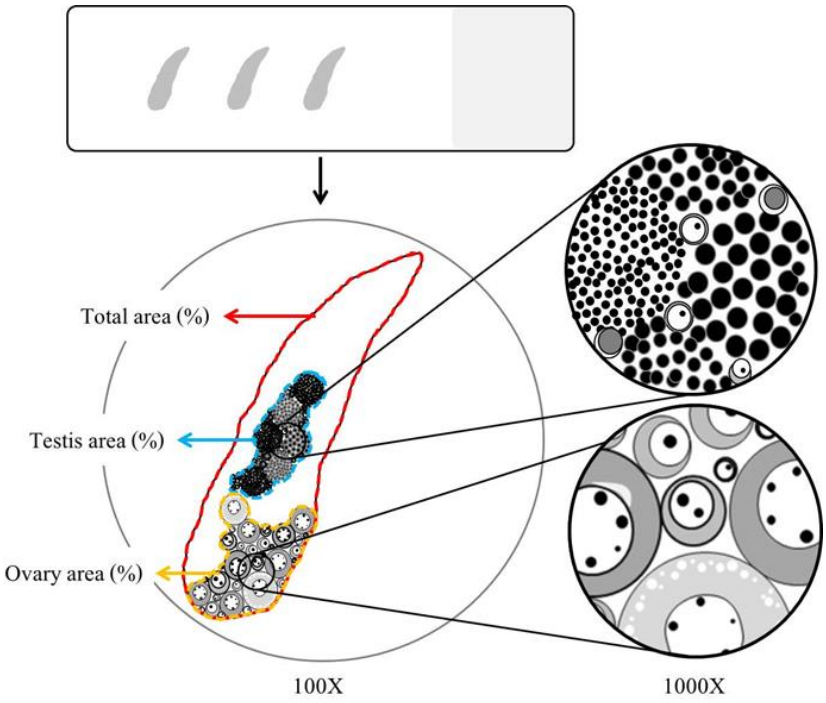

Fig. 1 Schematic representative of the percentage measurement of the differentiated gonads of false clown anemonefish *Amphiprion ocellaris*.

10X RT Buffer, 2 µl of 20 U RNase Inhibitor, 1 µl of MultiScribe™ Reverse Transcriptase (Thermo Fisher Scientific, USA), and DEPC-treated water to a final volume of 20 µl. All tubes were placed in a 2720 Thermal Cycler (Applied Biosystems, USA) with the following cycling conditions: 65 ºC for 5 min, 25 ºC for 10 min, and 37 ºC for 30 min. The reaction was terminated by incubation at 85 ºC for 5 min and 4 ºC for 30 min. The cDNA was then stored at -20 ºC until used.

Quantitative real-time PCR (qRT-PCR)

The primer pairs for the target *igf1* gene and the *18S rRNA* reference gene, with product sizes of 141 and 102 bp, respectively, were as follows: *igf1* (Forward: 5′-GATGTGCTGTATCTCCTG-3′, Reverse: 5′-CTCTCTCCACAGACAAAC-3′, Tm (ºC): 86.38, E (%): 105, R^2: 0.99) and *18S rRNA* (Forward: 5′-CGAGGAATTCCCAGTAAG-3′, Reverse: 5′-CTCACTAAACCATCCAATC-3′, Tm (ºC): 80.94, E (%): 99, R^2: 0.98). Primer sequences were designed using Primer3 software (Untergasser et al. 2012) to target the conserved sequences of the *igf1*-specific and *18S rRNA* reference genes across teleost species. Following PCR amplification, the products were verified through sequencing and BLAST analysis against the GenBank database. The reaction efficiency of qRT-PCR for both genes was validated in triplicate using standard curves prepared from ten-fold serially diluted plasmid DNA. Efficiency was calculated from the regression slope of the assay: $E = (10^{-1/slope})-1$ (Svec et al. 2015), and ranged from 99 to 105%. The reproducibility of the assay (R^2) was consistent > 0.98.

The qRT-PCR reactions were performed with a total volume of 15 µl, which included 7.5 µl of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA), 0.5 µl of 10 µM forward and reverse primers, 1 µl of cDNA templates, and 5.5 µl of nuclease-free water. The qRT-PCR cycling conditions were as follows: 95 ºC for 10 minutes, then 40 cycles of 15 seconds (s) at 95 ºC, 20 s at 53 ºC, and 20 s at 72 ºC for both *igf1* and *18S rRNA* genes. After completing these steps, the temperature was incrementally raised by 0.3 ºC from 60 ºC to 95 ºC using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA).

For gene expression analysis, all 40 samples (i.e., 5 × 8 monthly fish samples) were prepared in triplicate, along with serial dilutions of plasmid DNA, to create standards for real-time PCR baseline determination. A melting curve analysis was included to estimate the assay's analytical specificity, whereby only a single PCR amplicon was detected. No DNA template-controlled reaction was included in the qRT-PCR test. The quantification of *igf1* transcripts was normalized to the *18S rRNA* gene using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), as described by Boonphakdee et al. (2019). The mean value and standard deviation were calculated from triplicate samples.

Statistical analyses were performed using IBM SPSS Statistics 24 software. The homogeneity of variance was tested using Levene's test, and data that did not conform were log-transformed. One-way analysis of variance (ANOVA) was used to compare the expression levels of the target *igf1* and reference *18S rRNA* genes at different time points. Post-hoc multiple comparisons were conducted using the Duncan test. Expression values with $P < 0.05$ were considered significant, and data are reported as the mean ± standard error of the mean.

Ethics statement

All fish investigations and procedures in this study complied with the guiding principles for using and caring for animals for scientific purposes Act, B.E. 2558 (National Research Council of Thailand). The Burapha University Research Ethics Committee sanctioned the study (Ethic Code No: 31/2559).

Results

Fish growth

The growth of the false clown anemonefish from juvenile to sub-adult stages (i.e., n = 8 individuals sampled each month) revealed a significant increase in mean total length and weight as the fish aged. The mean total length of the fish increased from 21.01 ± 0.75 mm at one month to 43.64 ± 3.26 mm at eight months, while the mean weight increased from 0.18 ± 0.02 g to 1.53 ± 0.29 g (data not shown). The length-weight rela-

tionship of the experimental false clownfish aged one to eight months old displayed a curvilinear pattern. A strong correlation was found between body weight and total length ($R^2 = 0.9809$). The relationship was described by the equation $W = 0.000038L^{2.8015}$, where the value of b (2.8015) differed significantly ($P < 0.05$) from the standard value of 3.0, indicating a negative allometric growth pattern (Fig. 2A). This suggests that the fish did not undergo proportional changes in body weight as they increased in total length.

During the 30 days between their first and second months of age, the fish exhibited a notably high SGR of total length, with an average SGR of 0.27 ± 0.04% day^{-1}. The SGR calculated from the body weight also demonstrated a similar pattern, with a mean SGR of 0.82 ± 0.10% day^{-1} in the same initial 30-day period (data not shown). The growth rates, however, declined over the experimental period for both growth measures. The SGR of total length decreased from 0.27 ± 0.04% day^{-1} to 0.13 ± 0.01% day^{-1} for the 7- and 8-month-old fish (Fig. 2B). Similarly, the specific growth rate of body weight decreased from 0.82 ± 0.10% day^{-1} to 0.38 ± 0.03% day^{-1} in the same period (data not shown). These declines indicate that the fish experienced reduced growth rates as they aged beyond their early developmental stages.

Fish gonadal histological analysis

During the first two months, the gonads were undifferentiated, and primordial germ cells (PGCs) were identified above the hindgut (pgc; Fig. 3Aa-e). These PGCs were characterized by a clear border demarcating the cytoplasm from the nucleus, a strong staining with hematoxylin, and a close association with blood vessels (bv) (Fig. 3Ac). By the third month, ovarian differentiation in certain areas of the gonads was noted, signified by the presence of oogonia (og) and chromatin nucleolus (cn) stage oocytes (Fig. 3Ba-b). This demonstrated the commencement of early oogenesis in the anemonefish.

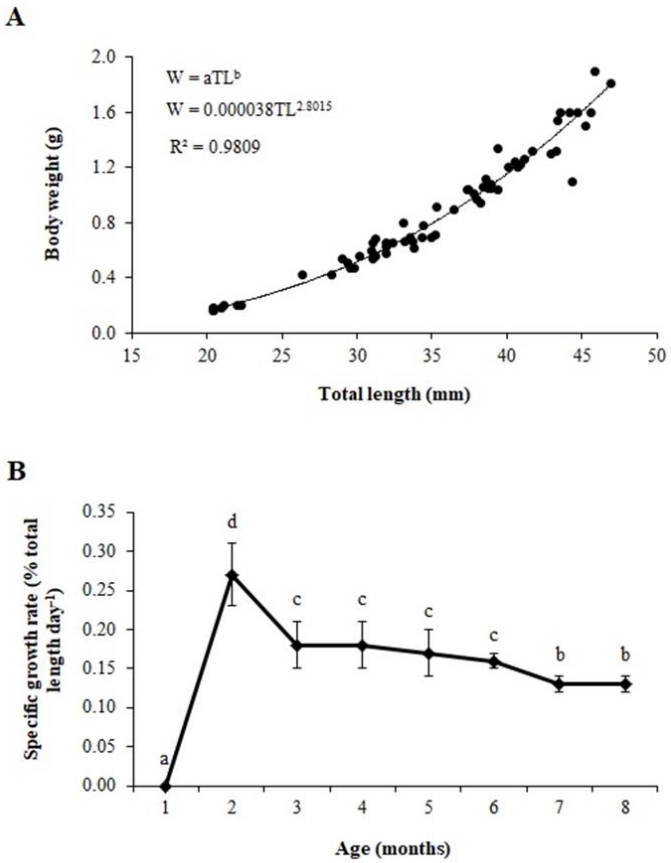

Fig. 2 Length-weight relationship (LWR) and specific growth rate (SGR) of captive-reared false clown anemonefish (*Amphiprion ocellaris*) aged one to eight months. (A) LWR shows the growth pattern of the fish, with their weight plotted against their length. The relationship appears to follow a negative allometric growth, indicating that as the length of the fish increases, its weight increases proportionally less than its length. (B) SGR (% day^{-1}) reveals a significant ($P < 0.05$) higher SGR in terms of total length at two months old compared to the other age groups (n = 8 fish at each time point; mean ± SD).

At the 4-month mark, oocyte differentiation was predominantly visible at the chromatin nucleolus and oogonia stages (Fig. 3Bc). Intriguingly, within the same individual fish, an evolution in the development of male germ cells was observed, including spermatogonia (sg), spermatocytes (1sc, 2sc), spermatids (st), and a small number of spermatozoa (sz) – the latter representing the final and smallest stage of male germ cell development (Fig. 3Ca). This implies simultaneous oogenesis and spermatogenesis within individual anemonefish at 4 months of age.

From the fourth month to the eighth, anemonefish gonads experience ovotestis formation. However, only the primary growth stage of oocytes persists in the female germ cell development. The oogonia, chromatin nucleolus, and perinucleolus stages of oocytes are dispersed throughout the gonadal tissue (Fig. 3Bc, d). When the anemonefish reaches 8 months of age, perinucleolus (pn) oocytes are more abundant than other oocyte stages. These oocytes exhibit a round shape, possess a large nucleus, have a less basophilic

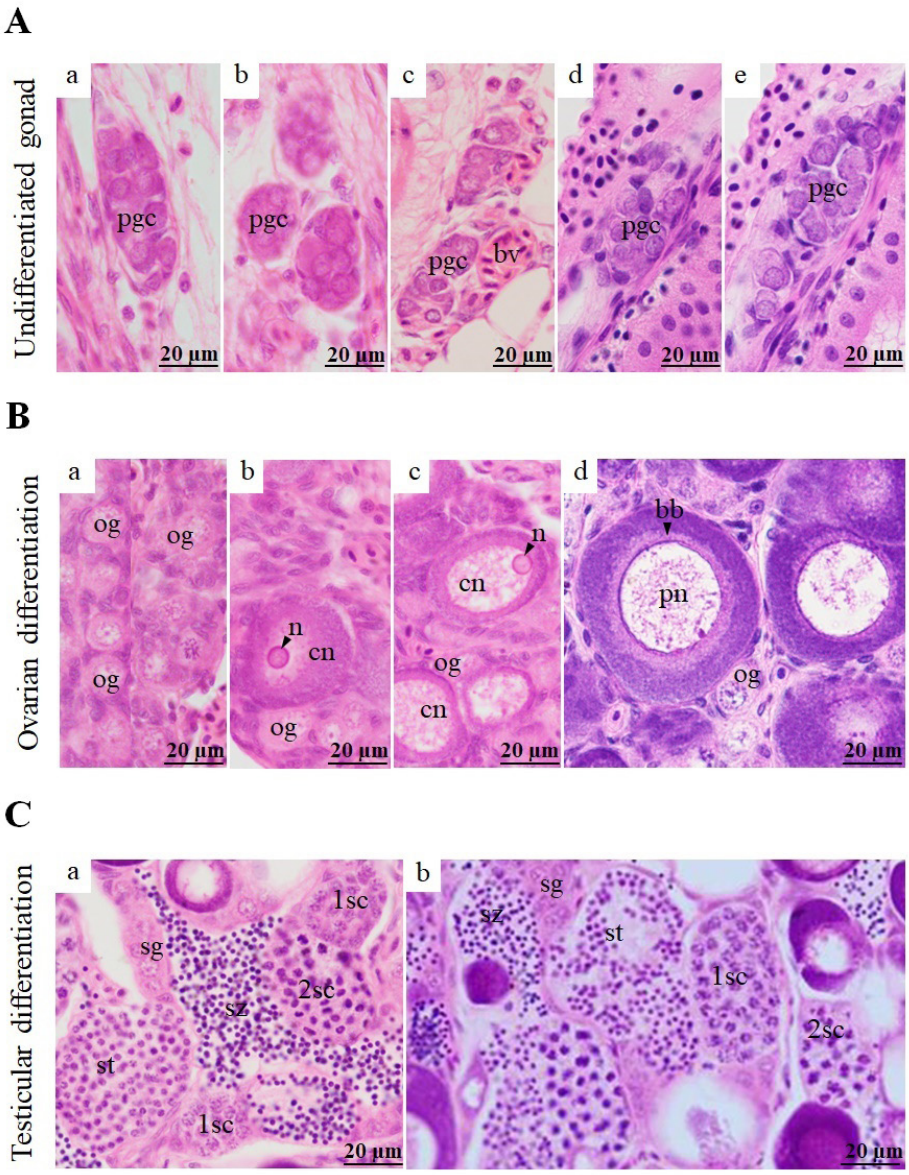

Fig. 3 Histological analysis of developing gonads of captive-reared false clown anemonefish (*Amphiprion ocellaris*). Longitudinal sections of gonads in one- to eight-month-old-aged fish. (A) Undifferentiated gonads in one- to two-month-old fish featuring primordial germ cells surrounded by somatic cells (Aa-e) and blood vessels near PGCs (Ac). (B) Ovarian differentiation at three- to eight-months-old, showcasing the oogonium (Ba), the chromatin nucleolus stage (Bb, c), and the perinucleolus stage (Bd). (C) Testicular differentiation, displaying the presence of spermatogonia, spermatocytes, spermatids, and spermatozoa (Ca, b) in four- to eight-month-old fish. All histological sections were stained with hematoxylin and eosin. Abbreviations: bb = Balbiani's vitelline body, bv = blood vessel, cn = chromatin nucleolus stage, n = nucleolus, og = oogonia, pgc = primordial germ cell, pn = perinucleolus stage, 1sc = primary spermatocyte, 2sc = secondary spermatocyte, sg = spermatogonia, st = spermatid, sz = spermatozoa.

and irregularly contoured cytoplasm, and possess conspicuous Balbiani vitelline bodies (bb; as shown in Fig. 3Bd). The nucleus of these cells manifests a mild decrease in basophilia. This phase, however, does not exhibit oocytes' previtellogenic (cortical alveoli) and vitellogenic stages. In contrast, the increase in the number of male germ cells interspersed with female germ cells within the gonadal tissue directly correlates with the age of the fish. The development of the male germ cells at all stages - including spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa - accelerates markedly when compared to their state at 8 months of age (Fig. 3Cb).

The proportions of the undifferentiated gonad, ovarian, and testicular tissue in fish from 1 to 8 months old are illustrated in Fig. 4. The undifferentiated gonad was prevalent in the initial two months, represents the entire gonadal area, which then receded to 59.4 ± 0.6% by the third month. Throughout development, the gonadal area occupied by ovarian tissue consistently surpassed that of testicular tissue. The ovarian tissue progressively increased in size, constituting 40.6 ± 0.5%, 45.5 ± 0.5%, 86.0 ± 0.4%, 83.9 ± 0.3%, 83.1 ± 0.6%, and 82.6 ± 0.3% of the total gonadal area in fish from the third to the eighth month, respectively. Conversely, the testicular tissue occupies a smaller proportion of the gonadal area. It increases from covering a mere 1.8 ± 0.3% of the total gonadal area at 4 months old to 17.4 ± 0.4% by the time the fish are 8 months old.

Quantification of *igf1* mRNA transcripts in liver and gonadal tissues

This study revealed a differential regulation of *igf1* expression in the liver and gonads of false clownfish during early development and growth. The liver *igf1* expression remained relatively stable between one and six months of age, with only minor fluctuations. A subtle decrease in expression was observed in 1- to 2-month-old clownfish, reaching its lowest point at 2 months old (0.26 ± 0.04). A modest increase in expression occurred in fish aged 3 to 6 months. Importantly, a significant increase ($P < 0.05$) in *igf1* expression levels was detected from 7 months onwards, peaking in 8-month-old fish (1.87 ± 0.18). Conversely, the gonadal *igf1* expression profiles displayed a markedly different pattern. The highest expression levels were observed in 2-month-old clownfish (478.17 ± 25.88), followed by a significant decline ($P < 0.05$) in fish aged 3 months and older. The lowest expression levels were identified in 7-month-old clownfish (0.68 ± 0.10; Fig. 5).

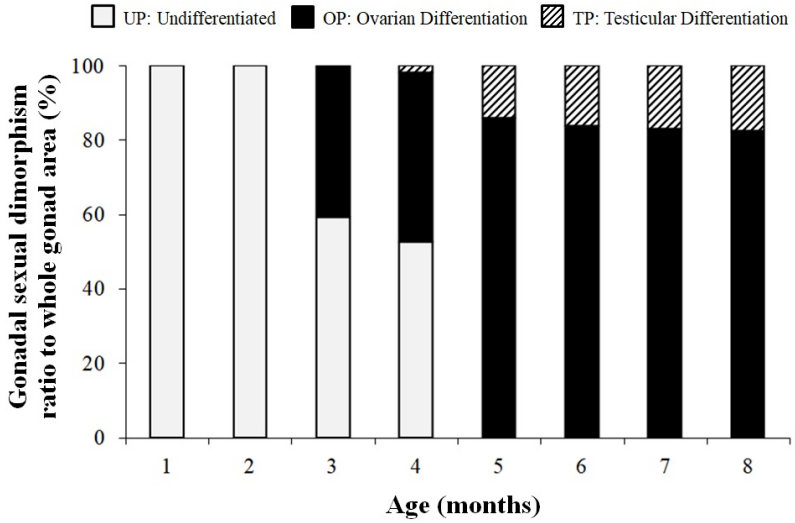

Fig. 4 Differential gonadal development in false clown anemonefish (*Amphiprion ocellaris*) aged between 1 and 8 months. The percentage proportion of undifferentiated gonads (UP), ovarian tissue (OP), and testicular tissue (TP) at various stages of development in false clown anemonefish based on histological analysis is illustrated. The percentages were calculated by determining the surface area ratios of female (or male) gonadal tissue to the overall gonad area. The histological analysis encompassed three out of the eight fish randomly sampled each month. For each fish, nine serial longitudinal histological sections were captured from the gonadal center. The presence of primordial germ cells and blood vessels distinguishes undifferentiated gonads (UP). The ovarian differentiation phase (OP) is characterized by features such as blood vessels, an ovarian cavity, oogonia, primary-growth stage oocytes, and a range of spermatogenic stages. Similarly, the testicular differentiation phase (TP) is marked by attributes including blood vessels, an ovarian cavity, oogonia, primary-growth stage oocytes, and diverse stages of spermatogenesis.

Discussion

This study investigated the growth patterns and gonadal development of captive-reared false clown anemonefish aged one to eight months. To minimize the influence of social factors on growth patterns, the two largest and the smallest fish in each tank were excluded from each monthly sample. The study identified a distinct growth pattern, with the mean total length of the fish increasing from 21.01 ± 0.75 mm to 43.64 ± 3.26 mm and their mean weight rising from 0.18 ± 0.02 g to 1.53 ± 0.29 g. These findings are consistent with the results of Madhu et al. (2010, 2012) and Abol-Munafi et al. (2011) regarding juvenile and non-breeding false clownfish. Histological examination of the gonads in the current study revealed an abundance of perinucleolus oocytes, indicating an immature stage in false clownfish before their subsequent sex change (Madhu et al. 2010).

We observed a negative allometric growth pattern in our captive-reared anemonefish based on the relationship described by the equation $W = 0.000038L^{2.8015}$, b = 2.8015). This was consistent with other studies on different fish species (Fox et al. 2010; Thulasitha and Sivashanthini 2013; Tesfahun 2018). This pattern, however, contradicts the findings of Khoo et al. (2018) who studied false clownfish in their natural habitat; the differences between the two studies may be due to differences in the life stages studied and the environmental conditions. Variations in b values, indicative of growth patterns, can be attributed to age, sex, season, temperature, diet, and genetics (Khoo et al. 2018; Mohamed et al. 2018). Environmental conditions and food resources greatly influence these growth patterns (Brett 1979; Houde 1997). Stress levels, sexual maturation rates, and genetic factors also contribute to the observed differences between captive-reared and wild-caught false clownfish (Bonga 1997; Barton 2002; Gjedrem 2005; Jobling 2010; Cooke et al. 2013).

Distinct patterns of *igf1* mRNA expression have been observed in the liver and gonadal tissues of false clownfish during early development and growth. In the liver, *igf1* mRNA remains stable from one to six months but experiences a significant increase in the seventh month, reaching its peak in eight-month-old

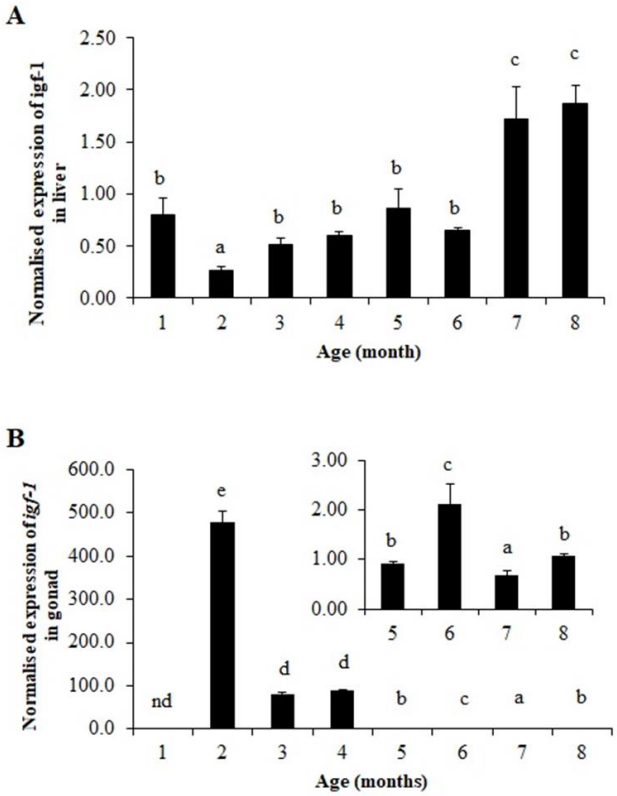

Fig. 5 Expression levels of *igf1* in false clown anemonefish (*Amphiprion ocellaris*) aged one- to eight months old. Expression levels of the *igf1* mRNA transcripts were measured in fish using qRT-PCR. (A) Expression of *igf1* in the liver, (B) *igf1* in the gonads (the inset graph displays data for five- to eight-month-old fish). The values were calibrated with the reference *18S rRNA* gene according to the cycle threshold (CT) method ($2^{-\Delta\Delta CT}$ method), as detailed by Livak and Schmittgen (2001). Data are presented as the mean ± SEM (n = 5 for each value). Different letters indicate statistical differences (P <0.05) between age groups. nd = denotes no data available.

fish. Conversely, the highest expression level in gonadal tissues is observed in two-month-old clownfish, followed by a notable decline in fish aged three months and older. The lowest expression levels are found in seven- and eight-month-old clownfish. The inverse relationship between *igf1* expression levels and growth rate suggests that *igf1* expression may be influenced by the growth stage or size of the fish rather than directly determining the growth rate itself. These dynamic expression patterns of *igf1* in the liver and gonadal tissues indicate its potential involvement in later stages of growth and development, potentially contributing to the observed reduction in growth rate during maturation (Duran et al. 2022). A study on pacu (*Piaractus mesopotamicus*) revealed that IGF1 regulates muscle growth, as indicated by transcriptome and microRNAome analysis (Duran et al. 2022).

Histological analysis of false clownfish gonads indicates their initial undifferentiated state. By the third month, early oogenesis begins with the appearance of oogonia and early-stage oocytes, signifying ovarian differentiation and a concurrent decline in undifferentiated gonadal tissues. Notably, in the fourth month, a unique phenomenon emerges with subsequent simultaneous oogenesis and spermatogenesis, resulting in the formation of an ovotestis. This ovotestis persists from the fourth to the eighth month, accompanied by the expansion of primary oocytes and increasing male germ cell numbers. These observations, which are consistent with prior research (Miura et al. 2003; Rattanayuvakorn et al. 2006; Iwata et al. 2008; Abol-Munafi et al. 2011), reinforce the robustness of our findings.

The progression of oogenesis corresponds to a gradual rise in primary oocyte numbers, extending until the eighth month. This evidence lends additional credence to our hypothesis of *igf1*'s role in overseeing ovarian development and maturation in false clownfish. Importantly, our findings align with the review of Li et al. (2015) and Ndandala et al. (2022), highlighting IGF-1's presence in fish ovarian developmental pathways. Furthermore, our study unveils a noteworthy discovery, i.e., the development of an ovotestis, distinguished by a proportionately larger amount of ovarian tissue in fish aged four to eight months. This insight seamlessly integrates into the context of *A. ocellaris*' protandrous life cycle, a well-documented phenomenon involving the male-to-female transition. This life cycle pattern is a prevailing trait within this species, offering significant insights into its intricate reproductive dynamics. Additional identifying specific binding sites for IGF-1 in fish reproductive tissues suggests that IGF-1 is involved in hormone production and oocyte maturation during fish gonadal development. Further studies, including the manipulation of plasma IGF-1 levels, employing larger sample sizes, and encompassing a wider range of fish ages, would provide more definitive insights and support this hypothesis.

While this study does not directly measure plasma IGF-1 levels, further investigations are warranted to understand better the molecular mechanisms underlying gonadal differentiation and the roles of hormones, including IGF-1, IGF-2, and IGF-3. The IGF-2 hormone, another member of the IGF family, has been shown to stimulate thymidine incorporation in goldfish vitellogenic follicles and promote oocyte maturation in various fish species, such as red seabream (*Pagrus major*) and mummichog (*Fundulus heteroclitus*) (see Reinecke 2010b; Rotwein 2018). The dependence of IGF-2 on GH in teleost fish, however, remains controversial (Ndandala et al. 2022). The expression of IGF-1 and IGF-2 have been identified in ovarian follicle cells of many fish species during the vitellogenic phase, potentially influencing ovarian steroidogenesis. In the ovaries of yellowtail (*Seriola quinqueradiata*), IGF-1 and IGF-2 have been found to facilitate the conversion of progesterone and 17α-hydroxyprogesterone to androstenedione, potentially regulating their ovarian development and maturation (Higuchi et al. 2020). On the other hand, the third member, IGF-3, is specific to certain fish species such as zebrafish (*Danio rerio*), Nile tilapia (*Oreochromis niloticus*), Atlantic hagfish (*Myxine glutinosa*), medaka (*Oryzias latipes*), and sea lamprey (*Petromyzon marinus*) (see Wang et al. 2008; Fox et al. 2010; Ndandala et al. 2022; Xie et al. 2022). IGF-3 primarily exhibits expression in the gonads and initiates early in fish development, while other teleost fish and elasmobranchs possess IGF-1 and IGF-2. The precise role and function of IGF-3 in fish biology, however, remains unclear.

Further investigations are warranted to explore the contribution of additional factors and hormones to gonadal sexual development, providing a more comprehensive understanding of their interplay with IGF-1. Specifically, research should focus on the proteins or genes involved in the synthesis of sex hormones, including female 17β-estradiol (E2), male testosterone (T), and 11-ketotestosterone (11-KT), which are known to play crucial roles in germ cell proliferation and differentiation in fish gonads (Nader et al. 1999; Iwata et al. 2008; Wang et al. 2020). The study by Nader et al. (1999) supports the potential involvement of recombinant human IGF-1 in stimulating various stages of spermatogenesis in the Japanese eel, *Anguilla*

japonica, *in vitro*. By investigating these factors, we can gain deeper insights into the intricate mechanisms underlying gonadal development and maturation in fish, which would have practical implications for enhancing the breeding and rearing of captive fish.

Conclusions

In conclusion, this study provides valuable insights into the growth and reproductive development of the false clown anemonefish, a hermaphroditic species. Our findings reveal a negative allometric growth pattern and provide important information on the timing of gonadal differentiation during the first eight months of the fish's life. The expression patterns of *igf1* mRNA transcripts in the liver and gonadal tissues suggest the involvement of IGF1 in ovarian differentiation and the growth of primary oocytes, highlighting its crucial role in gonadal development and maturation. Further investigations, however, are needed to comprehensively explore the interplay between *igf1* mRNA transcripts and other regulatory factors, the manipulation of plasma IGF-1 levels, and elucidate the specific functions of IGF-2 and IGF-3 to gain a deeper understanding of the molecular mechanisms underlying fish gonadal development. Such investigations will contribute to our knowledge of reproductive biology in fish and have practical implications for the captive management of clownfish.

Competing interests The authors declare that they have no competing interests.

Authors' contributions CB conceived the study, SS, JT, and CB conducted the histology and quantification of the *igf1* mRNA transcripts by quantitative real-time PCR. SS, JT, CB, and APS contributed to interpreting the results. SS led in writing the manuscript in consultation with JT, CB, and APS.

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