Changes in mRNA abundance of insulin-like growth factors in the brain and liver of a tropical damselfish, Chrysiptera cyanea, in relation to seasonal and food-manipulated reproduction

Changes in mRNA abundance of insulin-like growth factors in the brain and liver of a tropical damselfish, *Chrysiptera cyanea,* in relation to seasonal and food-manipulated reproduction

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Running head: Involvement of IGF in tropical damselfish reproduction

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Abstract

Food availability can become a factor driving the reproductive activity of tropical fish, particularly when primary production within their habitats fluctuates with tropical monsoons. The present study examined the involvement of insulin-like growth factors (IGF) in controlling the reproduction of the sapphire devil *Chrysiptera cyanea*, a reef-associated damselfish that is capable of manipulating its reproductive activity based on food availability. We cloned and characterized the cDNAs of *igf1* and *igf2* and determined their transcript levels in relation to seasonal and food-manipulated reproduction. The cDNAs of sapphire devil *igf1* and *igf2* had open reading frames (ORFs) composed of 600 bp (155 amino acid residue) and 636 bp (165 aa), respectively. Phylogenetic analyses revealed that IGF1 and IGF2 of the sapphire devil were clustered into those of teleosts. The gonadosomatic index increased from March to June. Vitellogenic oocytes and ovulatory follicles were observed in ovaries from May to June, which suggests that the spawning season lasts for at least 2 months. The hepatosomatic index, but not the condition factor, increased in March and June. The transcript levels of *igf*s in the brain, but not in the liver, increased in April/May (peak vitellogenesis) and July (post vitellogenesis). Ovarian activity during the spawning season was maintained by high food supply (HH) for 4 weeks, although it was suppressed in the food-restriction treatment (LL) and restored in the re-feeding treatment (LH). The transcript levels of *igf*s in the brain, but not in the liver, in LH were lower than those in HH and LL. Moreover, immersing fish in seawater containing estradiol-17β suppressed transcript levels of *igf*s in the liver, but not in the brain. We conclude that reproductive activity during the spawning season is influenced by nutritive conditions and that crosstalk exists between the reproductive and growth network in the neural and peripheral tissues, thus controlling the reproductive activity of this species.

Keywords: Coral Reef, Damselfish, Food availability, Insulin-like growth factor, Tropical

54 monsoon, Vitellogenesis

Introduction

Reproductive success in fish is closely related to adaptive ability under various environmental conditions. In general, the principal environmental factor affecting the seasonal reproduction of temperate fish is photoperiod (Bromage et al., 2001; Pankhurst and Porter, 2003); long days initiate and accelerate gonadal development in long-day spawners (Björnsson et al., 1998), while short days cue reproductive activity in short-day spawners (Masuda et al., 2005). The interaction between photoperiod and water temperature is also reportedly involved in the initiation and termination of seasonal reproduction in certain fish (Pankhurst and King, 2010; Shimizu et al., 2003).

A transitional shift in the proximate factor controlling the reproductive activity of fish may occur from high to low latitude, due to minimal fluctuations of photoperiod and temperature in tropical waters (Ohga et al., 2015). Previous studies have reported that the goldlined spinefoot *Siganus guttatus* inhabiting coral reefs off of the Okinawa Islands, Japan (subtropical waters; 26°42’ N, 127°52’ E), exhibits one spawning season lasting 2 months from June to July (Rahman et al., 2000), while the same species inhabiting coral reefs off of the Karimunjawa archipelago, Indonesia (tropical waters; 05°83’S, 110°46’E), exhibits its main spawning season from September to November and a minor one from March to May (Sri Susilo et al., 2009). In the former case, reproductive activity is likely cued by periodical changes in photoperiod and temperature, as it begins in concert with annual increases in these environmental factors (Takemura et al., 2015). On the other hand, reproduction in the latter case is initiated during transition periods between the rainy and dry seasons, which suggests the involvement of additional factors related to periodical changes in tropical monsoons (Sri Susilo et al., 2009). Johannes (1978) proposed that in addition to temperature, rainfall, and the speed of prevailing currents and winds, plankton productivity can also initiate reproductive activity in tropical species. In a field survey, Tyler and Stanton (1995) revealed that the

reproductive activity of the green damselfish *Abydefduf abdominalis* in Kaneohe Bay, Hawaii, was positively correlated with stream discharge. Because the reproductive activity of this species is restored by feeding (Tyler and Stanton, 1995), food availability in regional waters becomes a possible driver governing the reproductive ability of fish at the population level within a habitat. This concept may be applicable to other tropical species; for example, the spawning season of the millet butterflyfish *Chaetodon miliaris* is correlated with the productivity of calanoid copepods (Ralston, 1981). These findings raise the hypothesis that an interplay exists between the reproductive and growth endocrine axes, although the physiological mechanisms of how growth factors, including leptin, glucocorticoid, and insulin-like growth factor (IGF), modulate the neuroendocrine systems remains unknown in fish (Zohar et al., 2010). Fluctuation of the growth endocrine system with changes in food intake may drive the reproductive activity of tropical fish under suitable ranges of principal environmental determinants.

The sapphire devil *Chrysiptera cyanea* is a tropical damselfish belonging to the family Pomacentridae and is commonly distributed within the West Pacific region (Myers, 1999). Previous studies of the sapphire devil in coral reefs around the Okinawa Islands have demonstrated that vitellogenesis in the female starts in March and peaks in May (Bapary et al., 2009), and that spermatogenesis in the male starts in March and actively undergoes from April to May (Igarashi et al., 2015). It has been experimentally shown that the progress of vitellogenesis could be induced under long-day conditions with a suitable temperature range during the non-spawning season (Bapary et al., 2009; Bapary and Takemura, 2010) and controlled by food supply during the spawning season (Bapary et al., 2012). These previous contributions imply that the sapphire devil represents an ideal species for studying how growth factors are involved in the initiation and termination of reproductive activity in fish. We document the involvement of growth factors in controlling the ovarian development of

105 the sapphire devil to better understand the interplay between the growth and reproductive
106 network of tropical fish. We focused on IGF in particular, because it is a peptide hormone that
107 belongs to the growth factor family and is involved in metabolism, cell regeneration, and
108 proliferation in many organisms (Reinecke et al., 2005). IGF also plays an important role in
109 physiological processes including body growth, embryonic development, and reproduction
110 (Li et al., 2015; Reinecke, 2010), although there is limited knowledge regarding how growth
111 factors including IGF modulate the neuroendocrine system in fish (Zohar et al., 2010). We
112 measured the transcript levels of *igf*s in the brain and liver of the sapphire devil in relation to
113 seasonal reproduction and food-manipulated reproduction. Effects of estradiol-17β (E2)
114 treatment on the transcript levels of *igf*s in these tissues were also evaluated. Two isoforms of
115 *igf*s (*igf1* and *igf2*) of this species were cloned and characterized, and their transcript levels
116 were determined using real-time quantitative polymerase chain reaction (qPCR).
117
118 **Materials and Methods**
119 Fish and experimental design
120 The sapphire devils used in the present study (0.43 to 4.17 g in body mass) were collected
121 from Iri-jima (26°15'26.2" N 127°41'13.8" E), Okinawa, Japan, during daytime low tide
122 using a seine net. They were either sampled immediately at the Department of Chemistry,
123 Biology and Marine Science, University of the Ryukyus, Nishihara, Japan, or reared in stock
124 tanks at Sesoko Station, Tropical Biosphere Research Center (TBRC), University of the
125 Ryukyus, Motobu, Japan, until the onset of experiments. All experiments were conducted in
126 compliance with the Animal Care and Use Committee guidelines of the University of the
127 Ryukyus and regulations for the care and use of laboratory animals in Japan.
128 The first experiment (Experiment 1) examined seasonal changes in reproductive activity
129 as well as the involvement of nutritive status in reproductive activity of the sapphire devil in

Okinawa. Just after monthly collection of fish at Irijima, matured females (n = 7–8 per month) were anaesthetized with 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan). After recording body mass and total length of each individual, the fish were sacrificed by decapitation. The entire brain including the pituitary was separated from the skull. Then the ovary and liver were removed from the abdominal cavity, and their masses were recorded. One lobe of the ovary was preserved in Bouin's solution for histological observation. The gonadosomatic index (GSI = [ovarian mass/body mass] × 100), the hepatosomatic index (HSI = [liver mass/body mass] × 100], and condition factor (K = [body mass /total length^3] × 100) were calculated. The whole brain and pieces of the liver and ovary were homogenized in 500 μL RNAiso plus total RNA (Takara Bio, Otsu, Japan) and then stored at -80°C until further molecular analyses.

The second experiment (Experiment 2) was conducted from May to June 2016, to study the effects of food availability on nutritive status and reproductive activity in accordance with experimental protocols described previously (Bapary et al., 2012). Briefly, mature fish (24 females and 1–2 males per aquarium) were housed in three 60 L glass aquaria with running seawater and aeration under ambient water temperature and photoperiod at Sesoko Station. Plastic pipes were placed onto the bottom of the aquarium as a substrate and nest for territorial males in order to reproduce natural conditions. During acclimatization for 6 days, fish were fed commercial pellets (Pure Gold EP1; Nisshin-Marubei, Tokyo, Japan) at 5% of body mass daily at 10:00 h. Afterwards, fish in two aquaria were maintained on a daily supplement of food at 0.2% of body mass (continuous low food conditions; LL) or 2% of body mass (continuous high food conditions; HH) for 30 days. Fish in the residual aquarium were reared under low-food conditions (0.2%) for 15 days and then high-food conditions (2%) for 15 days (low to high food conditions; LH). At days 0, 15, and 30 after the start of the experiment, females (n = 7–8) were collected from each aquarium, anesthetized with

2-phenoxyethanol, and decapitated (Fig. 4A). Subsequent procedures for tissue preparation are as described for Experiment 1.

The third experiment (Experiment 3) was conducted to determine the effects of estradiol-17β (E2) treatment on the mRNA abundance of *igf1* and *igf2* in the liver and brain, and vitellogenin (*vtg*) in the liver. According to the previous studies (Imamura et al., 2017; Tong et al., 2004), E2 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethanol at a concentration of 1 mg/mL. Immature fish (0.46 to 1.13 g in body mass) were collected in August 2017 and then housed in three 30 L glass aquaria with aerated seawater at ambient temperature. After acclimatizing under rearing conditions, the fish (10 per aquarium) were exposed to E2, which was added to the seawater of two aquaria at final concentrations of 0.5 ng/mL (low-dose group) and 5 ng/mL (high-dose group). Vehicle was added to the residual aquarium (control group). After 3 days, the fish were removed from each aquarium, anesthetized with 2-phenoxyethanol, and sample collection occurred as described for Experiment 1.

Histological analyses

Following dehydration in a graded ethanol series and permutation with xylene, pieces of the ovary were embedded in histoparaffin (Paraplast Plus, Sigma-Aldrich, St. Louis, MO, USA), sectioned at 7 μm, and then stained using hematoxylin and eosin for microscopic observation. Oocytes in the ovaries were classified into the peri-nucleolus (PNS), oil-droplet (ODS), primary yolk (PYS), secondary yolk (SYS), and tertiary yolk (TYS) stages, according to the oocyte staging of the white-spotted spinefoot *Siganus canaliculatus* (Hoque et al., 1998). Post-ovulatory follicles (POFs) and atretic oocytes (AOs) were also observed following methods described elsewhere (Matsuyama et al., 1988).

Cloning and characterization of sapphire devil *igf1* and *igf2* cDNAs

Total RNA was extracted from the brain, liver, and ovary using RNAiso Plus Total RNA (Takara Bio), according to the manufacturer's protocol. RNA concentrations were checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed to synthesize cDNA from 70 ng total RNA using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio), according to the manufacturer's protocol.

The primer sets for sapphire devil *igf1* and *igf2* (Table 1) were designed based on the highly conserved regions of *igf1* and *igf2* sequences of *Stegastes partitus* (**XM_008280881** and **XM_008293672**, respectively). Partial fragments of sapphire devil *igf1* and *igf2* were amplified via PCR, with 30 cycles of denaturation (45 s at 94°C), annealing (45 s at 60°C), and extension (1 min at 72°C). PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into JM109 competent cells (Takara Bio). After each PCR product was checked by electrophoresis in 2% agarose (Takara Bio), samples were sent to Macrogen Japan (Kyoto, Japan) to determine DNA sequences using a 3730xl DNA analyzer (Applied Biosystems, Waltham, MA, USA).

The open reading frame (ORF) of sapphire devil *igf1* and *igf2* nucleotide sequences was identified and then translated into amino acids using a Web-based ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Then the identified ORFs were checked using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the identity of each sequence. The verified amino acid sequences of sapphire devil *igf1* and *igf2* were aligned by including other closely related teleosts as well as several additional taxa as outgroups using ClustalW (Thompson et al., 1994). Then the aligned sequences were constructed into a phylogenetic tree using maximum likelihood methods with the Whelan and Goldman (WAG) model of evolution approach (Whelan and Goldman, 2001) and 1,000 bootstrap replications.

205 The sequence alignment and phylogenetic construction were performed in MEGA 6.06
206 (Tamura et al., 2013).
207 The tissue distribution of *igf1* and *igf2* was checked using reverse transcription
208 (RT)-PCR under the following conditions: 30 cycles of denaturation (45 s at 94°C), annealing
209 (45 s at 60°C), and extension (1 min at 72°C). PCR products were electrophoresed in 2%
210 agarose gel containing ethidium at 110V for 20 min and visualized under UV.
211
212 Real-time quantitative PCR (qPCR)
213 The mRNA abundance of sapphire devil *igf1* and *igf2* in the liver and brain and sapphire devil
214 *vtg* (GenBank accession no. **LC383743**) in the liver was assayed using the CFX96 real-time
215 PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) and an SYBR Green
216 premix PCR kit (Takara Bio). Primer sets for detecting target genes are shown in Table 1.
217 Each PCR was carried out in a final volume of 10 μL containing 5 μL SYBR Premix Ex Taq
218 II (Tli RNaseH Plus) (Takara Bio), 0.3 μL forward and reverse primers, 2.4 μL nuclease free
219 water, and 2 μL cDNA template. The PCR conditions were as follows: denaturation (30 s at
220 95°C), 39 cycles of denaturation (5 s at 95°C), and annealing and extension (30 s at 60°C),
221 which had a melting point from 65 to 95°C with an incremental increase of 0.5°C each 5 s. A
222 melting curve analysis was performed subsequently to ensure single amplicon amplification.
223 The specific primer assays were performed using serial dilutions of liver cDNA and exhibited
224 amplification efficiencies close to 100%. The mRNA abundance of target genes in each
225 sample was normalized to the amount of *ef1α* as an internal control.
226
227 Statistical analyses
228 Data are expressed as means ± standard error of the mean (SEM). One-way analysis of
229 variance (ANOVA) and Kruskal-Wallis non-parametric analyses were applied according to

Barlett's homogeneity and the Shapiro-Wilk normality test. Multiple pairwise analyses using Tukey's honestly significant difference (HSD) test were applied to compare means among analyzed groups.

Results

Molecular cloning of sapphire devil *igf1* and *igf2*

The cDNAs of sapphire devil *igf1* (**LC383743**) and *igf2* (**LC383744**) both had ORFs, which were composed of 600 bp (155 amino acid residual) and 636 bp (165 amino acid residual), respectively. They each contained five domains (namely, the B-, C-, A-, D-, and E-domains), which are also found in the ORFs of other teleosts (data not shown). Phylogenetic analyses revealed that IGF1 and IGF2 of the sapphire devil were exclusively clustered with those of other teleost species (Fig. 1).

The tissue-specific expression of sapphire devil *igf1* and *igf2* was examined using RT-PCR. The mRNA expressions of these genes were detected in the brain, liver, and ovary. No amplified products were detected in the negative control (Fig. 2).

Changes in reproductive and growth parameters (Experiment 1)

Changes in environmental factors (water temperature and photoperiod) and body parameters of the sapphire devil from March to July in 2016 are shown in Table 2. During sample collection, the photoperiod increased from March (11:59 h) to June (13:46 h) and then decreased in July (13:37 h). Water temperature steadily increased from March (20.14 ± 0.22 °C) to June (27.33 ± 0.28 °C).

The mean value of GSI was 1.14 ± 0.10 in March. Values increased thereafter and peaked in June (7.25 ± 0.64). The GSI significantly ($P < 0.05$) decreased in July (0.96 ± 0.02). The highest value of HSI was recorded in March (2.58 ± 0.20) and then decreased. An increase

255 was recorded again in June (2.06 ± 0.19), and subsequently, HSI values decreased to basal
256 levels in July. The K value fluctuated within ranges between 1.97 ± 0.04 in March and 1.52 ±
257 0.09 in July. Values of K significantly ($P < 0.05$) decreased from March to July.

258 All ovaries in January were immature and contained only oocytes at PNS (Fig. 3a).
259 Vitellogenic oocytes at PYS and TYS were first observed in ovaries in March (Fig. 3b) and in
260 June (Fig. 3c), respectively. No vitellogenic oocytes were observed in ovaries in July,
261 although they were occupied by atretic oocytes and immature oocytes at PNS (Fig. 3d).

262 Transcript levels of sapphire devil *igf1* and *igf2* in the liver and brain were assessed using
263 qPCR (Table 2). Compared to transcript levels in March, significant increases in sapphire
264 devil *igf1* and *igf2* in the liver were observed in April and June, respectively. In the brain,
265 these significantly increased in April/July and June, respectively.

266

267 Effect of food availability on reproductive activity (Experiment 2)

268 Experiment 2 was conducted in May–June, when the sapphire devil undergoes active
269 reproduction (Table 2). The HH group had high GSI values during the experimental period.
270 The GSI of the LH and LL groups decreased at 15 days after the initiation of the experiment.
271 GSI remained at low levels when low levels of food were provided for another 15 days (LL).
272 When fish in the LH group were re-fed with high levels of food, the GSI increased
273 significantly ($P < 0.05$) and reached the level of the HH group (Fig. 4B). The HSI of the HH
274 group remained high throughout the experiment. Food limitation caused a decrease in the HSI
275 of the LL group. However, re-feeding resulted in an increase in the HSI of the LH group (Fig.
276 4C). By contrast, values of K did not vary among the three treatments (Fig. 4D).

277 The ovaries of the sapphire devil under different feeding regimes were observed
278 histologically. Oocytes at TYS were observed in ovaries of the HH group. When food was
279 limited, ovaries were at immature stages at PNS (LL and LH groups) at day 15. The same

ovarian condition was observed in the LL group at day 30. However, re-feeding resulted in the appearance of vitellogenic oocytes at TYS in ovaries of the LH group at day 30 (Table 3). The same result has already been reported in a previous study (Bapary et al., 2012).

Effect of food availability on *igf1* and *igf2* in the liver and brain

Transcript levels of sapphire devil *igf1* and *igf2* in the liver and brain were compared among the HH, LL, and LH groups using qPCR (Fig. 4). No significant differences in transcript levels were observed in the liver (Fig. 4E and G). On the other hand, the levels of *igf1* in the brain of the LH group was significantly lower ($P < 0.05$) than that of the HH group (Fig. 4F). A similar pattern was observed for the transcript level of *igf2* (Fig. 4H).

Effects of E2 treatment on *igf1*, *igf2*, and *vtg* in the liver and brain

Immature fish were immersed in seawater containing E2 to evaluate the effects of this reproductive steroid on the transcript levels of *igf1* and *igf2* in the liver and brain The levels of both in the liver decreased significantly ($P < 0.05$) when E2 was added to seawater at final concentrations of 0.5 and 5.0 ng/ml (Fig. 5A and C). By contrast, this treatment did not alter transcript levels of either gene in the brain (Fig. 5B and D).

Effect of E2 treatment on the transcript levels of *vtg* in the liver was also conducted in this study. Significantly higher induction of *vtg* was observed, when the fish were treated with E2 at 0.5 and 5.0 ng/ml (Fig. 5E).

Discussion

The cDNAs of sapphire devil *igf1* and *igf2* were successfully cloned, providing evidence that the alignment of the deduced amino acid sequences had highly conserved characteristics of IGF and that IGF1 and IGF2 are phylogenetically clustered with those of teleosts (Chen et al.,

2001; Li et al., 2012; Pérez et al., 2016; Reinecke and Loffing-Cueni, 1997; Schmid et al., 1999).

Bapary et al. (2009) documented the annual reproductive cycle of female sapphire devils in Okinawan waters, demonstrating that vitellogenesis initially begins in March and actively continues from April through June. Our results are consistent with that, although a slight difference in the peak month of GSI was recorded (peaks in May and June in the previous and present studies, respectively). Because vitellogenesis can be artificially induced in this tropical species under long-day conditions within a suitable range of water temperature (Bapary and Takemura, 2010), photoperiod clearly acts as the proximate determinant for reproduction. The values of HSI recorded in the present study increased twice in March and June, which coincide with the time periods of the initial increase in and the peak of GSI, respectively. Therefore, the correlation between these two parameters is likely to be related to the progression of reproductive events in this species. In the case of the Atlantic sardine *Sardina pilchardus*, a group-synchronous spawner, the HSI and GSI of males inversely fluctuated, while the HSI of females increased twice during months both in and out of the reproductive season, which suggests a dual function of the liver in females, namely, lipid metabolism and vitellogenin synthesis (Nunes et al., 2011). The latter function has been clearly documented in the rainbow trout *Oncorhynchus mykiss* (van Bohemen et al., 1981) and the red porgy *Pagrus pagrus* (Aristizabal, 2007), in which HSI increases concomitantly with the progression of vitellogenesis. Because increases in the HSI of the sapphire devil corresponded to phases of initial and peak vitellogenesis, a cross-link likely exists between liver function and reproductive performance, including vitellogenesis. The present study also showed that an increase in HSI is closely related to food intake, and consequently, reproductive performance, as food limitation caused significantly low values of HSI and

329 gonadal retraction, whereas re-feeding restored high values of HSI and rapid growth of
330 oocytes with yolk accumulation (Fig. 5).

331 Transcript levels of sapphire devil *igf1* and *igf2* in the brain, but not in the liver,
332 increased from March to July. These expression profiles imply that *igf*s play an autocrine and
333 paracrine role in regulating ovarian function, including the active and post phases of
334 vitellogenesis. Although we did not evaluate localization of *igf*s in the brain,
335 immunoreactivity against IGF-1 has been observed in Purkinje cells and dendrites in the
336 cerebellum as well as neurons throughout the brain of the Mozambique tilapia *Oreochromis*
337 *mossambicus* (Reinecke and Loffing-Cueni, 1997). In addition, several reports have
338 demonstrated that pre-incubation with IGF-1 leads to increases in GnRH-induced FSH release
339 from and FSH content in the cultured pituitary cells of immature coho salmon *O. kisutch*
340 (Baker et al., 2000) and in the pituitaries of the zebrafish *Danio rerio* (Lin and Ge, 2009) and
341 the masu salmon *O. masou* (Morita et al., 2006). On the other hand, IGF-1 enhances
342 LH-induced aromatase activity and P450arom gene expression in cultured ovarian follicles of
343 the red sea bream *Pagrus major* (Kagawa et al., 2003). Vitellogenesis in the sapphire devil
344 may be partially driven by IGF-activated gonadotrophs, although we did not evaluate whether
345 IGFs from neural (brain) and peripheral (liver) origins are involved in this process.

346 The present study demonstrated that the reproductive status of the sapphire devil is
347 strongly influenced by short-term trials of food availability; satiation maintained high
348 reproductive activity for 4 weeks (HH group), whereas ovaries retracted due to food limitation
349 for 2 weeks and vitellogenic oocytes disappeared from ovaries (LL and LH groups). This
350 condition lasted until the end of the experiment in the LL group. By contrast, subsequent
351 re-feeding for another 2 weeks in the LH group restored ovarian conditions, and many
352 oocytes laden with yolk appeared in the ovaries. Because the HSI of the LL group was
353 significantly lower than that of the HH and LH groups 2 weeks after the initiation of the

experiment, fish reared under low-food conditions are certain to be malnourished. In fact, in a previous study, following trials of food limitation, the mRNA of leptin was upregulated in the liver of the goldlined spinefoot (Mahardini et al., unpublished data), and fasting caused upregulation of this peptide in the brain and liver of the mandarin fish *Siniperca chuatsi* (Yuan et al., 2016). In addition, yolk accumulation in each ovarian oocyte in our study was clearly influenced by changes in nutritional status. On the other hand, food limitation did not alter condition factor of the LL group. This result seemed to be different from the previous report, in which food limitation resulted in low condition factor in the female sapphire devil (Bapary et al., 2012). In this regard, since GSI in the HH group in the present study was two times higher than that in the previous one, difference in condition factor between the females may be partially due to initial difference in reproductive activity (in other word, in nutritive condition).

Food availability failed to alter transcript levels of *igf1* and *igf2* in the liver of the sapphire devil. This result is inconsistent with previous reports, in which the abundance of *igf1* and *igf2* in the liver of juvenile hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) decreased after fasting for 21 days but increased with re-feeding (Picha et al., 2008). Similar results have been reported for the liver of gilthead seabream *Sparus aurata* fingerlings, in which reduction in hepatic *igf1* transcription was induced by starvation for 8 days and then restored by re-feeding for 22 days (Metón et al., 2000). The discrepancy between our results and previous studies may be partially attributable to the sexual maturity of the fish used in each experiment. Unlike immatures and fingerlings, it is possible that the reproductive network of mature sapphire devils is activated under suitable photoperiod and water temperature and that food availability causes a rapid endocrinological shift between the reproductive and growth systems. This insight is supported in part by several previous studies. For example, the abundance of growth hormone (*gh*) mRNA in the pituitary of the goldlined

379 spinefoot increases with starvation for 15 days and decreases with re-feeding (Ayson et al.,
380 2007). In addition, transcript levels of gonadotropin-releasing hormone (*gnrh1*) are
381 significantly lower in the brains of LL fish than in LH fish (Mahardini, 2017). It was reported
382 that the way of data normalization (copy number/total RNA vs total liver copy number/body
383 weight) is affected by hepatic *igf1* mRNA level in the hybrid striped bass (Picha et al., 2008).
384 This may take into consideration in the sapphire devil because a rapid change in HSI (liver
385 size) occurred by food availability.

386 The transcript levels of sapphire devil *igf1* and *igf2* in the brain of the LH group were
387 significantly lower than those of the HH group, although fish of both groups underwent active
388 vitellogenesis and their ovaries contained developing oocytes laden with yolk. In addition, the
389 transcript level of *igf2* in the brain of the LH group was lower than that of the LL group. To
390 date, little is known about autocrine and paracrine roles of *igf*s in the brain of fish. However,
391 the present results point to several possibilities: the high transcript levels of *igf*s are
392 maintained under continuous food supply (relevant to the reproductive system); they are not
393 influenced by food limitation (relevant to the growth system); and a rapid restoration of
394 reproductive activity by re-feeding (cross-link between the reproductive and growth systems).
395 Interestingly, Mahardini (2017) demonstrated that the transcript levels of *kiss2* remain low in
396 LH fish, although their reproductive activity returns to the levels of HH fish, which suggests
397 that the reproductive system is not fully restored by re-feeding.

398 We believe that the rapid progression of vitellogenesis after re-feeding in the LH group
399 was due to active E2 synthesis in ovarian follicles. Therefore, an E2 increase had the potential
400 to negatively impact the transcript levels of *igf*s in the liver and brain of the sapphire devil.
401 Our results clearly show that immersing fish in seawater containing E2 reduced the transcript
402 levels of *igf*s in the liver but not in the brain. Similar experiments in other fish species have
403 concluded that treatment with E2 downregulates hepatic IGF-1 expression (Carnevali et al.,

2005; Davis et al., 2007; Filby et al., 2006; Hanson et al., 2012; Lerner et al., 2007; Riley et al., 2004; 2002). One exception was reported when intraperitoneal injections of E2 (5 μg/g body weight) failed to alter the transcript levels of *igf1* and *igf2* in the liver of immature rainbow trout within 72 h (Weber, 2015). Riley et al. (2004) found that E2 (0.1 to 100 μM) stimulates vitellogenin release and inhibits IGF-I expression in cultured hepatocytes, and the authors suggested that a mechanism exists to redirect available metabolic energy away from somatic growth toward oocyte growth in female Mozambique tilapia. Therefore, re-feeding stimulates the redirection of metabolic energy in the liver of the sapphire devil, which was supported by our observation of induction of *vtg* abundance in the liver after E2 treatment. On the other hand, E2-regulated paracrine/autocrine function may exist in the brain of this species because some reports have indicated extrahepatic production and autocrine/paracrine function of IGF1 in fish (Eppler et al., 2007; Wuertz et al., 2007).

In conclusion, the initiation and continuation of reproduction are in part driven by the nutritive status of individual sapphire devils under ranges of photoperiod and water temperature that are suitable for reproduction. The growth system including GH/IGF is likely to serve as a driver in neural and peripheral tissues and interacts with the reproductive system in matured fish. However, careful interpretation may be also indispensable in this interaction because it is known that the activation and control of reproductive and nutritive system are different among stages (ca. immature, pubertal, and mature stage) and facing environments (Reinecke, 2010). Further studies are needed to examine roles of GH/IGF system through life cycle of the sapphire devil. In addition, it is necessary to evaluate the involvement of other growth factors in driving reproduction in this tropical fish.

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618

Table 1. Primes used in the present study.

Primer	Sequence
Cloning	
igf1-Forward	5'-GCGCTCTTTCCTTTCAG-3'
igf1-Reverse	5'-CTCGACTTGAGTTTTTC-3'
igf2-Forward	5'-AAACCCAGCAAAGACACGGA-3'
igf2-Reverse	5'-CAAAGTTGTCCGTGGTGAGC-3'
Real-time PCR	
igf1-Forward	5'-ACAGCGACACACAGACATGC-3'
igf1-Reverse	5'-TGTGCCCTTGTCCACTTTG-3'
igf2-Forward	5'-ATTTCAGTAGGCCGACCAGC-3'
igf2-Reverse	5'-TCCTGTTTTTAGTGCGGGCAT-3'
vgt-Forward	5'-CAACGAGGAAACCGTGCATG-3'
vtg-Reverse	5'-GTTGCGGTGACAGTGAGAGA-3'
ef1α-Forward	5'-ACGTGTCCGTCAAGGAAATC-3'
ef1α-Reverse	5'-GGGTGGTCAGGATGATGAC-3'

Table 2. Seasonal changes in environmental factors, body parameters, and transcript levels of *igf*s of the sapphire devil.

Parameters	Month*				
	March	April	May	June	July
Environmental factors					
Day-length (h)**	11:59	12:47	13:26	13:46	13:37
Water Temperature (°C)	20.14 ± 0.22	22.67 ± 0.18	24.65 ± 0.29	27.33 ± 0.28	29.3 ± 0.13
Body parameters					
Body Weight (g)	2.53 ± 0.27^{a}	1.35 ± 0.15^{b}	1.84 ± 0.21^{ab}	2.03 ± 0.17^{ab}	1.45 ± 0.05^{b}
Gonadosomatic Index	1.14 ± 0.10^{a}	1.87 ± 0.49^{ab}	4.43 ± 0.47^{c}	7.25 ± 0.64^{d}	0.69 ± 0.02^{b}
Hepatosomatic Index	2.58 ± 0.20^{a}	1.23 ± 0.08^{b}	1.43 ± 0.06^{b}	2.06 ± 0.19^{a}	1.11 ± 0.20^{b}
Condition Factor	1.97 ± 0.04^{a}	1.65 ± 0.09^{bc}	1.75 ± 0.03^{b}	1.74 ± 0.06^{b}	1.52 ± 0.09^{c}
Relative expression level in the liver					
igf1	0.19 ± 0.04^{ac}	2.37 ± 0.82^{bd}	1.13 ± 0.28^{ab}	0.22 ± 0.07^{cd}	1.22 ± 0.55^{abcd}
igf2	0.63 ± 0.08^{ab}	0.38 ± 0.06^{c}	0.26 ± 0.07^{c}	2.23 ± 0.45^{d}	1.85 ± 0.77^{acd}
Relative expression level in the brain					
igf1	0.98 ± 0.04^{a}	2.01 ± 0.39^{b}	1.63 ± 0.26^{b}	1.84 ± 0.20^{b}	3.68 ± 0.73^{c}
igf2	0.81 ± 0.15^{acd}	0.74 ± 0.15^{ac}	0.61 ± 0.09^{ab}	1.26 ± 0.17^{d}	0.65 ± 0.19^{abc}

*Different letters indicate significant difference at $P < 0.05$.
**Day-length is expressed as the value of the middle of each month.
***Water temperature is expressed as the median of each month.

Table 3. Comparison of oocyte composition in ovaries of the fish groups with different food supply.

Group*	Oocyte stages**					Atretic oocytes**
	PNS	ODS	PYS	SYS	TYS	
Day 0						
IC	++	+	++	+	++	–
Day 15						
HH	++	+	++	+	++	–
LH and LL	++	–	–	–	–	–
Day 30						
HH	++	+	++	+	++	–
LH	++	+	++	+	++	–
LL	++	–	–	–	–	++

*IC, HH, LH, and LL indicate the fish groups, which were initial control at Day 0, fed with high food for 30 days, fed with low food for 15 days and high food for another 15 days, and fed with low food for 30 days, respectively.

**PNS, ODS, PYS, SYS, and TYS are abbreviations of oocyte stage (see materials and methods). Presence (+ and ++) and absence (-) of oocytes were expressed as +/++ and –, respectively.

Figure legends

Figure 1. Phylogenetic tree of IGF1 and IGF2 sequences of vertebrates. Maximum likelihood analysis with 1000 bootstrap replications was performed to construct the tree. Each value under the node indicates the bootstrap proportion value (maximum proportion value = 100). The scale bar represents the substitution rates per site. Accession number of each reference is indicated as follows: *igf1* (**AB465576** *Takifugu rubripes,* **XM008280881** *Stegastes partitus*, **NM001303334** *Larymichtys crocea*, **AY996779** *Sparus aurata*, **AJ586907** *Perca fluviatalis*, **KC800696** *Leiostomus xanthurus*, **KF819506** *Rana sylvatica*, **NM001004384** *Gallus gallus*, **CR541861** *Homo sapiens*, **CT010364** *Mus muculus*, **NM001313855** *Canis lupus familiaris*, **JN315416** *Pantheropis guttatus*); *igf2* (**NM001279643** *Oreochromis niloticus*, **Y18691** *Oreochromis mossambicus*, **JN596879** *Lateolabrax japonicus*, **KT727923** *Trachinotus ovatus*, **AY552787** *Ephinepelus coioides*, **HM164111** *Siniperca chuatsi*, **EU283335** *Amphiprion clarkii*, **JQ398497** *Megalobrama amblycephala,* **AF250289** *Danio rerio*, **AY603685** *Bos taurus*, **NM010524** *Mus muculus*, **NM001030342** *Gallus gallus*, **AJ223165** *Zebra finch*, **NM001195825** *Canis lupus*, **NM001113672** *Xenopus tropicalis*). Multiple alignments of amino acid were performed using ClustalW in MEGA 6.06.

Figure 2. RT-PCR analysis of sapphire devil *igf1* and *igf2* expression. Total RNA was extracted from the brain, liver, and ovary of the sapphire devil and reversetranscribed. After the sapphire devil *igf1*, *igf2*, and *ef1α* in each tissue were amplified by PCR, products were electrophoresed. Negative control (N.C.) was also indicated.

Figure 3. Ovarian histology of the sapphire devil. a; Cross-section (SC) of an ovary in March, b; SC of an ovary in April, c; CS of an ovary in June, d; CS of an ovary in July. PNS; peri-nucleous stage, PYS; primary yolk stage, TYS; tertiary yolk stage, AO; atretic

oocytes. Scale bar = 100 μm.

Figure 4. Effect of different food availability on mRNA abundance of sapphire devil *igf1* and *igf2* in the liver and brain of females. (A) Experimental design of food availability (arrow heads indicate the points of sample collections), (B) GSI, (C) HSI, (D) K, (E) *igf1* in the liver, (F) *igf1* in the brain, (G) *igf2* in the liver, (H) *igf2* in the brain. Fish were acclimated with food supply at 5% of body mass daily at 10:00 h, and then divided into three groups. HH; food was given at 2% of body mass for 30 days. LL; food was given at 0.2% of body mass for 30 days. LH; food was given at 0.2% of body mass for 15 days and 2.0% of body mass for 15 days. Data were normalized by determining the amount of sapphire devil *ef1α* and each point was expressed as mean ± SEM. Different letters indicate significant difference at $P < 0.05$.

Figure 5. Effect of E2 treatment on mRNA abundance of sapphire devil *igf1*, *igf2,* and *vtg*. (A) *igf1* in the liver, (B) *igf1* in the brain, (C) *igf2* in the liver, (D) *igf2* in the brain, (E) *vtg* in the liver. Immature fish were immersed in E2 containing seawater at concentration of 0.5 and 5.0 ng/ml for 3 days and then sampled. Data were normalized by determining the amount of sapphire devil *ef1α* and each point was expressed as mean ± SEM. Different letters indicate significant difference at $P < 0.05$.

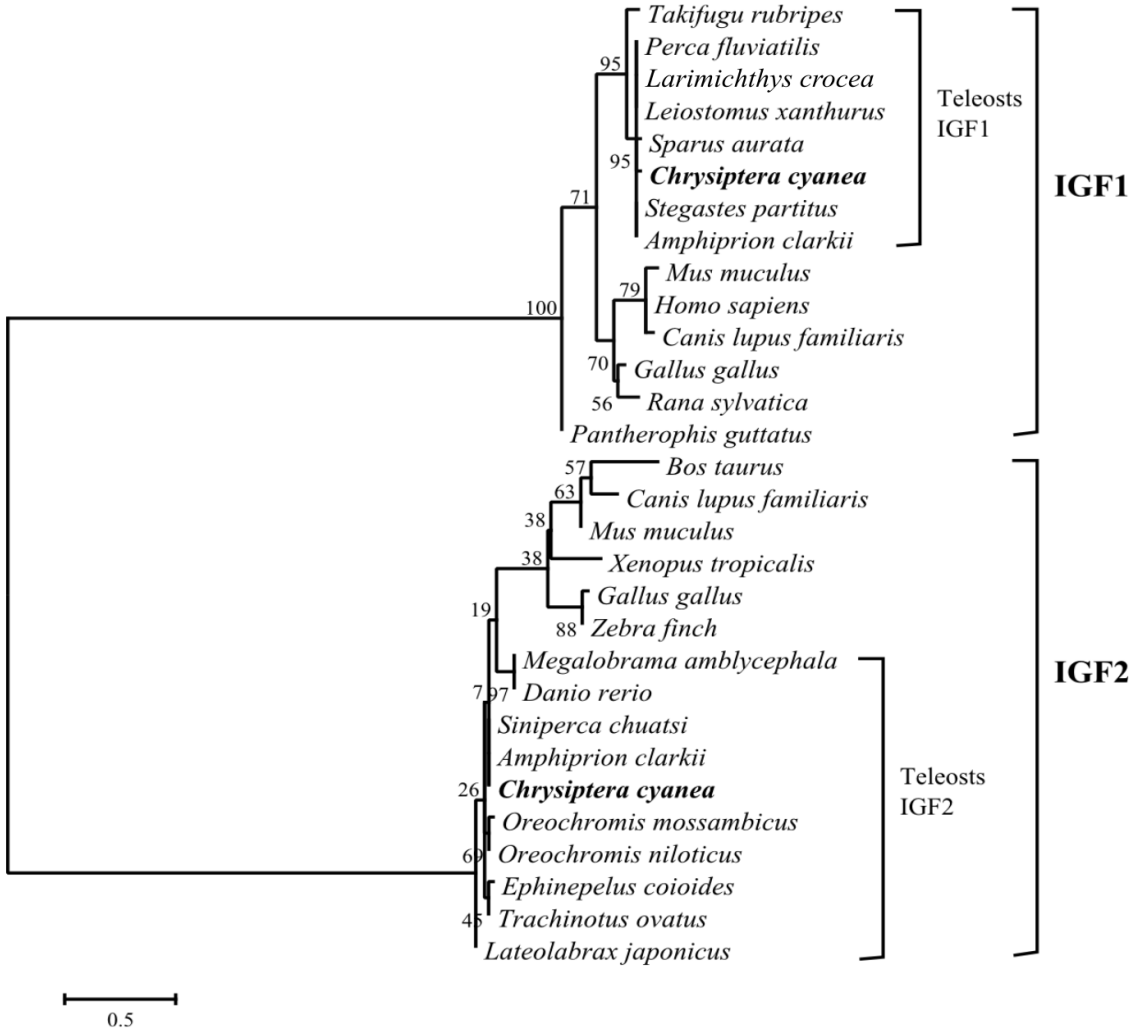
Takifugu rubripes
Perca fluviatilis
Larimichthys crocea
Leiostomus xanthurus
Sparus aurata
Chrysiptera cyanea
Stegastes partitus
Amphiprion clarkii
Mus muculus
Homo sapiens
Canis lupus familiaris
Gallus gallus
Rana sylvatica
Pantherophis guttatus
Bos taurus
Canis lupus familiaris
Mus muculus
Xenopus tropicalis
Gallus gallus
Zebra finch
Megalobrama amblycephala
Danio rerio
Siniperca chuatsi
Amphiprion clarkii
Chrysiptera cyanea
Oreochromis mossambicus
Oreochromis niloticus
Ephinepelus coioides
Trachinotus ovatus
Lateolabrax japonicus
95
95
71
100
79
70
56
57
63
38
38
19
88
7
97
26
69
45
Teleosts IGF1
IGF1
Teleosts IGF2
IGF2
0.5

Figure 1

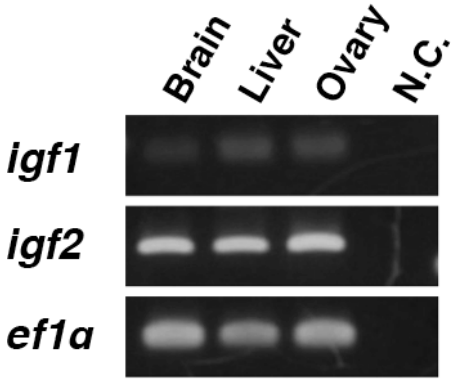

Figure 2

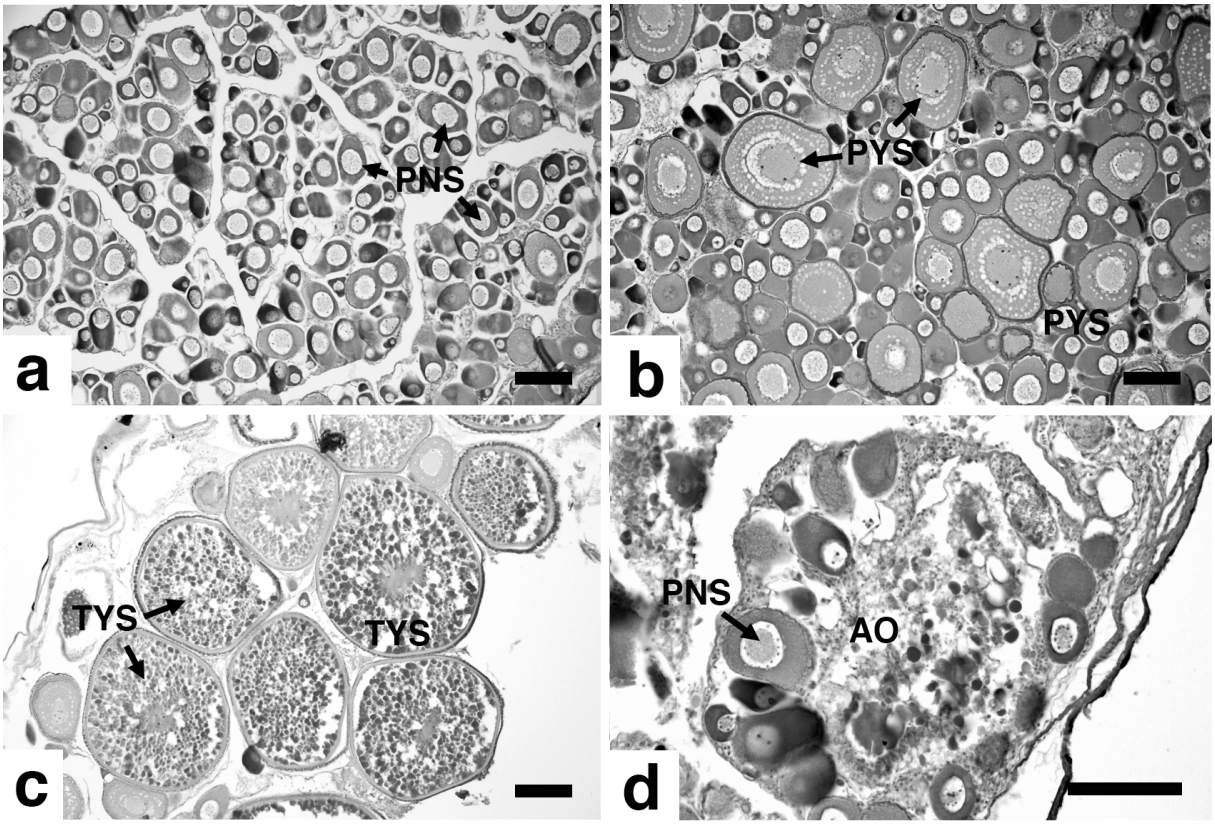

Figure 3

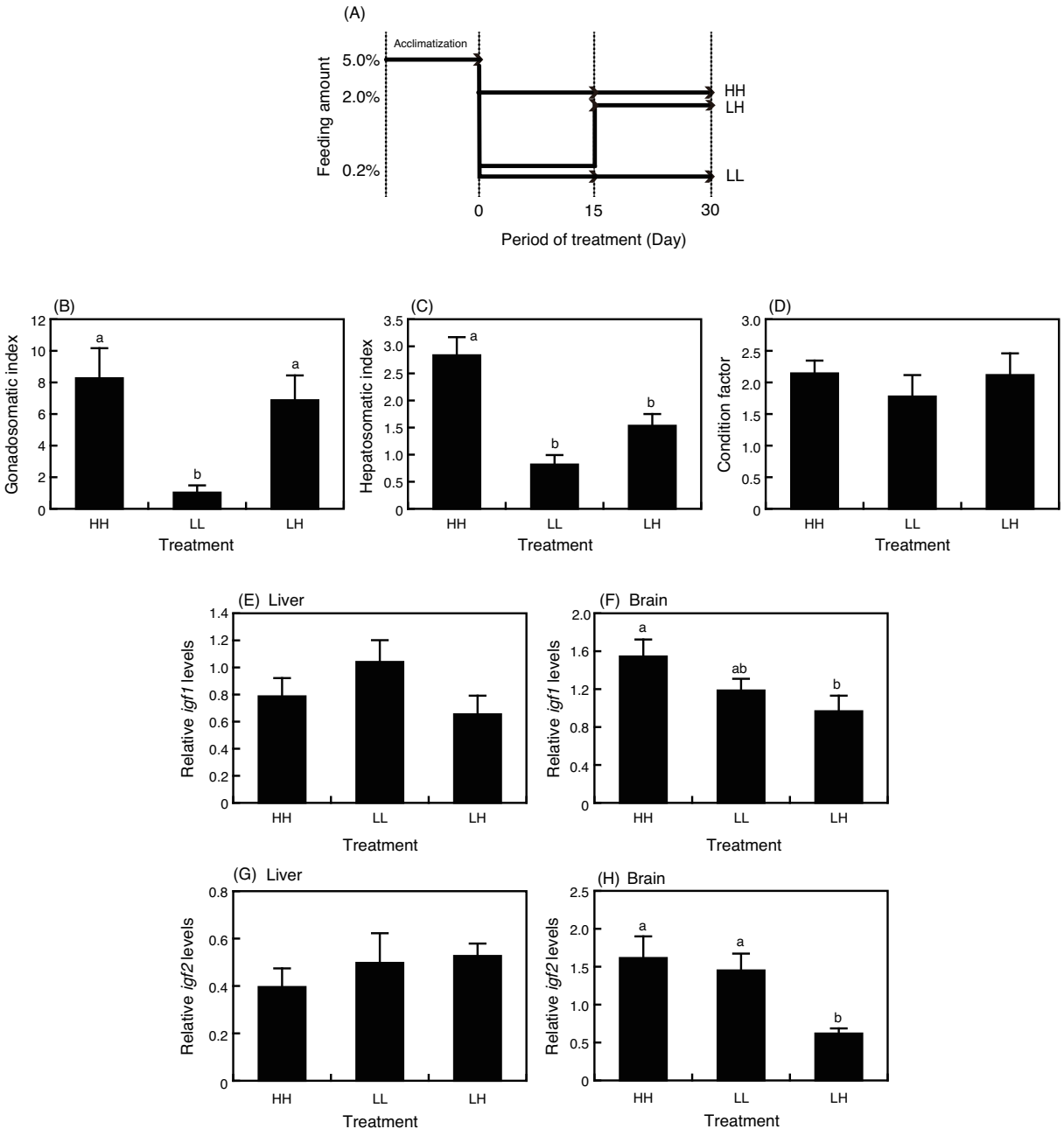
(A)
Acclimatization
5.0%
2.0%
0.2%
Feeding amount
HH
LH
LL
0
15
30
Period of treatment (Day)
(B)
Gonadosomatic index
a
b
a
HH
LL
LH
Treatment
(C)
Hepatosomatic index
a
b
b
HH
LL
LH
Treatment
(D)
Condition factor
HH
LL
LH
Treatment
(E) Liver
Relative igf1 levels
HH
LL
LH
Treatment
(F) Brain
Relative igf1 levels
a
ab
b
HH
LL
LH
Treatment
(G) Liver
Relative igf2 levels
HH
LL
LH
Treatment
(H) Brain
Relative igf2 levels
a
a
b
HH
LL
LH
Treatment

Figure 4

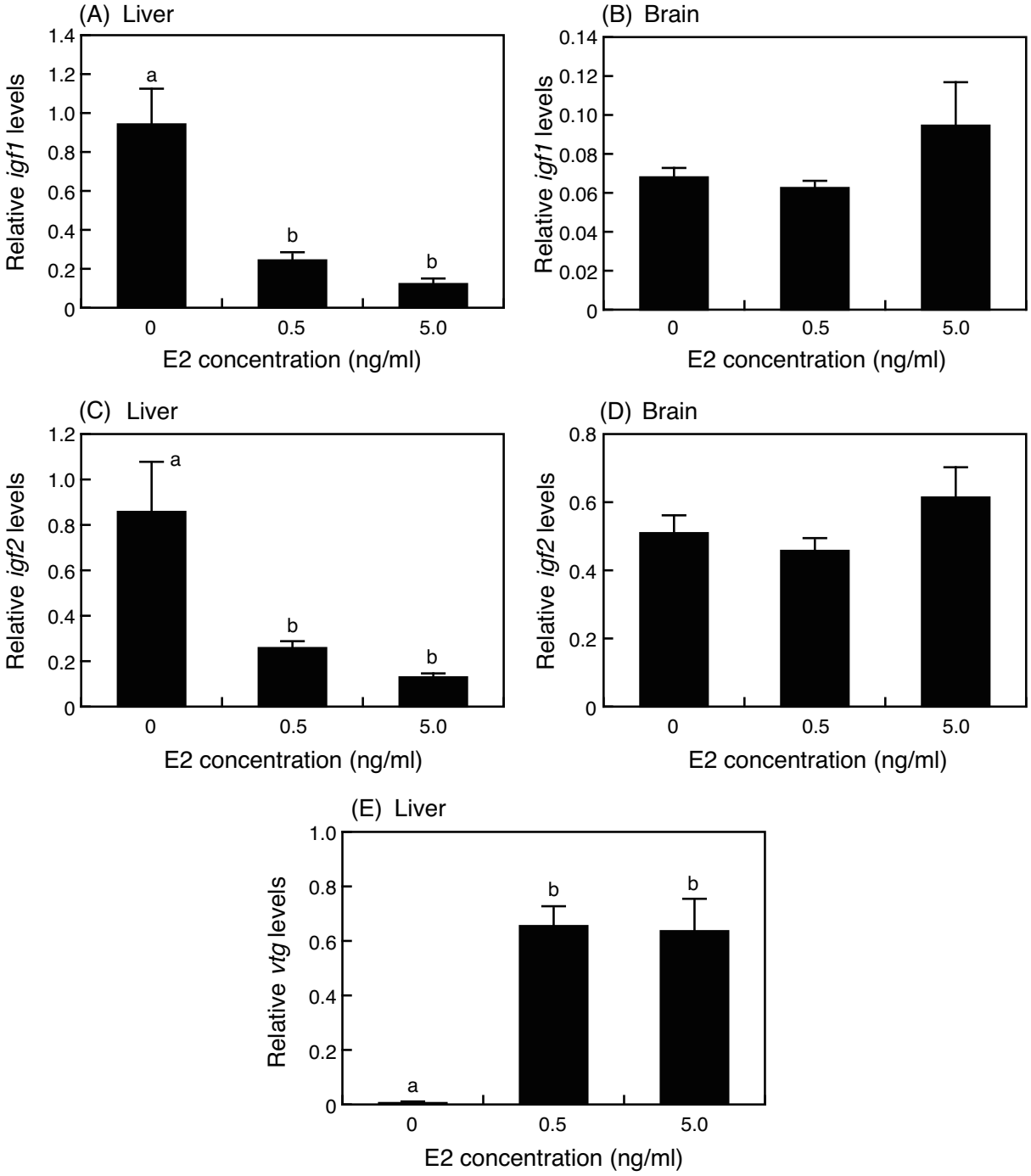
(A) Liver
Relative igf1 levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
a
b
b
0
0.5
5.0
E2 concentration (ng/ml)
(B) Brain
Relative igf1 levels
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0
0.5
5.0
E2 concentration (ng/ml)
(C) Liver
Relative igf2 levels
1.2
1.0
0.8
0.6
0.4
0.2
0
a
b
b
0
0.5
5.0
E2 concentration (ng/ml)
(D) Brain
Relative igf2 levels
0.8
0.6
0.4
0.2
0
0
0.5
5.0
E2 concentration (ng/ml)
(E) Liver
Relative vtg levels
1.0
0.8
0.6
0.4
0.2
0
a
b
b
0
0.5
5.0
E2 concentration (ng/ml)

Figure 5