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Source: Zoological Science, 29(12) : 849-855

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.29.849

ZOOLOGICAL SCIENCE **29**: 849–855 (2012)

Social Interaction Influences Blood Cortisol Values and Brain Aromatase Genes in the Protandrous False Clown Anemonefish, *Amphiprion ocellaris*

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Anemonefish, *Amphiprion* spp., are socially controlled, protandrous sex changers with a monogamous mating system. Under certain conditions, sexually immature anemonefish with ambisexual gonads differentiate directly into males or females. Formation and maintenance of social rank in a group are considered key requirements for the induction of sex change or differentiation. Generally, each animal living in a social group experiences a different level of social stress in accordance with its social rank, and we hypothesize that the stress situation of individual anemonefish influences its sex determination. Groups of three sexually immature anemonefish were placed into each of five experimental tanks and kept for 10 days to allow for social rank formation and behavioral observation. The fish were then euthanized, and blood and brain samples were collected from each fish. The social rank of each individual was distinguishable from day 1 of the experiment. Aggressive behaviors were most frequent and blood cortisol values were higher in dominant individuals. The transcription of mRNA for stress-related genes, i.e., those encoding for glucocorticoid and arginine vasotocin receptors, was higher in the brains of dominant individuals than in other social ranks. Furthermore, we detected higher transcription levels of gonad and brain aromatase genes, which encode the enzyme that converts androgens into estrogens, in the brains of dominant individuals. These results suggest that social rank reflects the blood cortisol value, which in turn leads to sex differentiation by manipulating transcription of genes, including aromatase genes, in the brain.

Key words: sex change, anemonefish, social behavior, stress, aromatase

INTRODUCTION

Plasticity in sex differentiation is common in teleost fish. In hermaphroditic teleost species, social interaction with conspecifics controls sex determination or sex change (Fishelson, 1970; Munday et al., 2006). Among sex-changing fish, anemonefish (genus *Amphiprion*) are unique in that they are socially controlled, protandrous sex changers with a monogamous mating system. They live symbiotically with sea anemones in the tropical waters of the Indo-Pacific region, forming a social unit consisting of a monogamous pair and several nonbreeders or juveniles. Females are the largest and dominant members of the social groups, displaying frequent aggressive behavior toward other group members. The second-ranked individuals become males, and the others remain as nonreproductive individuals. If a female disappears from a social unit, the male changes sex, and the largest of the nonbreeders becomes a functional male (Fricke and Fricke, 1977; Moyer and Nakazono, 1978). Meanwhile, under certain conditions, immature anemonefish with ambisexual gonads differentiate directly into males or females. For example, when juvenile anemonefish are raised together in captivity, the largest will become a female and the next largest a male, whereas the rest will remain sexually immature (Goldstein, 1989).

Anemonefish take about 45 d for a male-to-female sex change (Fricke, 1975; Godwin and Thomas, 1993) and several months or more for sex differentiation in an ambisexual pair (Iwata et al., 2008; Iwata et al., 2010a). Thus, the long-term social interaction in a group of anemonefish may be a key influence on the induction of sex change or differentiation (Iwata et al., 2008; Iwata et al., 2010a). In general, each animal living in a social group experiences a different level of social stress in accordance with its social rank (Goymann and Wingfield, 2004), and we hypothesize that the stress level of individual fish as determined by its social rank influences its sex determination.

The relationship between sex differentiation and physical stress, but not social stress, is well documented in gonochoristic teleost species with thermolabile sex determination (TSD), such as the Japanese flounder *Paralichthys olivaceus* and pejerrey *Odontesthes bonariensis*. High temperatures result in increased levels of the stress-related hormone cor-

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doi:10.2108/zsj.29.849

tisol in the blood, and high cortisol levels suppress transcription of the aromatase gene, which leads to masculinization of larvae (Kitano et al., 2001; Hattori et al., 2009). Aromatase is the enzyme that converts testosterone to estradiol (E_2), and is also considered to be a key enzyme of sex determination in vertebrates, including sex-changing fish (Gardner et al., 2005; Guiguen et al., 2010). Numerous studies have documented the relationship between aromatase and sex change. For example, aromatase inhibitors (AI) block the natural sex change and induce male function in the protandrous black porgy *Acanthopagrus schlegeli* (Lee et al., 2002; Wu et al., 2005) and increase plasma androgen and stimulate development of the testis in the protogynous honeycomb grouper *Epinephelus merra* (Bhandari et al., 2005). Aromatase also plays an important role in socially controlled sex-changing fish, including anemonefishes (Nakamura et al., 1994; Kobayashi et al., 2004; Kroon et al., 2005; Kobayashi et al., 2010).

Teleosts are known to have two types of genes for aromatase, *cyp19a1a* and *cyp19a1b*, which are predominantly expressed in the ovary and brain, respectively (Tchoudakova and Callard, 1998). Gonadal *cyp19a1a* is involved in the sex differentiation or sex change of teleosts mentioned above, whereas the brain *cyp19a1b* gene is also involved in local E_2 synthesis and is considered to be related to brain sexualization and plasticity (Le Page et al., 2010; Okubo et al., 2011), although the details of these functions remain unclear, especially as they relate to sex-changing fish.

To investigate the relationship between stress, social rank, and sex differentiation in anemonefish, we evaluated the early stages of social rank formation by examining blood cortisol values and behavioral traits in groups of three individual false clown anemonefish *A. ocellaris* with ambisexual gonads kept in a tank for 10 d. We then determined transcription levels of *cyp19a1a* and *cyp19a1b* genes in the brain. We also evaluated the stress-related glucocorticoid receptor (*GR*) and arginine vasotocin receptor (*AVTR*) genes in the brain.

MATERIALS AND METHODS

Animals

Captive-bred sexually immature *A. ocellaris* with ambisexual gonads at least 12 months post hatch (provided by Dr. T. Furuta, Environmental Science Research Laboratory, Central Research Institute of Electric Power Industry, Chiba, Japan) were kept in a 56-L tank with a recirculating water system at 25–26°C under natural light. The fish were held in groups of 30 to 50 to suppress sexual maturation until the experiment started. Fish were fed commercial pellets (Omega One Marine Flakes, Omega Sea, Ltd., Perry, OH, USA) daily throughout the experiment.

Three fish were moved to each of five 26-L experimental tanks and kept for 10 d for social rank formation and behavioral observation. A PVC pipe coupling (50 mm diameter) substituted for a host sea anemone and was placed at the center of each experimental tank as a shelter. Each individual fish in a tank was identified by differences in the white-striped pattern on its body, and each fish was classified as α (dominant), β (second ranked), or γ (subordinate) on the basis of behavioral observations on day 1 of the experiment. Specifically, an individual that occupied the shelter most of the time was identified as α, an individual with the second highest occupation time was identified as β, and the individual with the shortest occupation time was identified as γ (Iwata et al., 2008).

The experimental protocols used in this study followed the Iwaki Meisei University's *Policies Governing the Use of Live Vertebrate Animals* and the Japan Ethological Society's *Guidelines for Research on Animal Behavior*.

Behavioral analysis

Because anemonefish are diurnally active, we videotaped twelve 5-min observation periods during the light period, starting at 0800, 0830, 1000, 1030, 1200, 1230, 1400, 1430, 1600, 1630, 1800, and 1830 on days 1, 4, 7, and 10. On the video recordings, we observed each experimental fish for the following four behaviors: duration of shelter occupation, frequency of threatening other fish by lunging, frequency of being the target of lunging, and frequency of trembling, which is an appeasement behavior (Moyer and Bell, 1976). The time spent in the shelter was converted to a percentage of the total observation time.

Sample collection and measurements

On day 10 of the experiment, after the video recording was completed, fish were captured and euthanized with 300 ppm of MS222 (tricaine methanesulfonate; Sigma-Aldrich, St. Louis, MO, USA). All resident fish in a tank were captured at once using a hand net and then moved to a smaller tank with the dissolved anesthetic chemical. The anesthesia was introduced within 3 min of the start of capture. Total body length and weight were measured, and blood samples were collected from the caudal vessel of each fish by using a heparinized capillary tube and centrifuged immediately. The plasma was removed and stored at –20°C in a plastic tube until assay. The brains were extracted and soaked in 500 μL of RNA-stabilization solution (RNAlater; Applied Biosystems, Carlsbad, CA, USA) in a 1.5-mL microfuge tube and stored at –20°C for gene analysis.

Hormone assays

Ten microliters of each plasma sample was extracted using 2 mL diethyl ether and resuspended in 200 μL of enzyme immunoassay (EIA) buffer (Cayman Chemical, Ann Arbor, MI, USA), and the concentration of cortisol was measured following the manufacturer's instructions in using a commercially available EIA kit (Cayman Chemical). All concentrations were measured in triplicate. The inter- and intra-assay coefficients of variation were 7.6% and 8.4%, respectively.

Partial gene sequencing

Total RNA was purified by using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of RNA was performed with a ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA). cDNA was amplified by PCR with a Takara Ex Taq Reaction Kit (Takara Bio, Shiga, Japan). The total PCR reaction volume of 30 μL was composed of 3.0 μL 10× Ex Taq Buffer, 3.0 μL dNTP mixture, 2.1 pmol of each primer, 0.8 units Ex Taq, and 1.0 μL DNA solution containing 0.15 μg cDNA. The degenerate oligonucleotide primer sets used in the PCR are listed in Table 1. The PCR products were ligated into the pCR 2.1-TOPO vector by using TOPO TA Cloning (Invitrogen). Gene sequences were confirmed by DNA sequencing with an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems). The gene sequences were compared with all other known gene sequences by using the Basic Local Alignment Search Tool (BLAST). Similar DNA sequences were downloaded from the DNA Data Bank of Japan (DDBJ) and aligned with our sequences. The data were registered in the DDBJ/EMBL (the European Molecular Biology Laboratory) /GenBank databases and assigned the accession numbers AB597954 (for *GR*), AB597955 (for *AVTR*), AB597952 (for *cyp19a1b*), and AB597953 (for *cyp19a1a*).

Real-time PCR analysis

For real-time PCR assay, gene-specific oligonucleotide primers were designed on ProbeFinder software (Roche Applied Science,

Table 1. Oligonucleotide sequences used for cloning, and their functions.

Oligo	Sequence	Function	Reference
1	ATCNGSMGGAAGAACTGCCC	*GR* forward	–
2	AGCATYTCYGGRAACTCCAC	*GR* reverse	
3	TACTTCATCTTCTCCCTRAG	*AVTR* forward	–
4	GGGTTRCAGCAGCTGCTGTTGAG	*AVTR* reverse	
5	GACATCTCYAACAGACTSTTCC	*cyp19a1a* forward	Blazquez and Piferrer (2004)
6	GCHGCGATCASCATYTCCA	*cyp19a1a* reverse	
7	TATGGSAGCATTGYKCGGGTKTGG	*cyp19a1b* forward	Strobl-Mazzulla et al. (2005)
8	GGGCTGGAAGAAACGACTG	*cyp19a1b* reverse	Ezagouri et al. (2008)
9	CAATGGATCCGGTATGTGC	*β-actin* forward	Naito et al. (1998)
10	CGTTGTAGAAGGTGTGATGCC	*β-actin* reverse	

Table 2. Oligonucleotide sequences used for quantitative PCR, and their functions.

Oligo	Sequence	Function
1	GCCGTTGCACTGTTGGTAA	*GR* forward
2	TCCTGGCTCTTCCTCATGTC	*GR* reverse
3	CTTTCGTGATAGTTCTGGCGTA	*AVTR* forward
4	GCATCTGCACGATCAAAAAC	*AVTR* reverse
5	AGCCCAGGAGCTACAAGATG	*cyp19a1a* forward
6	TTTGTCTGCCTGCTCCATATC	*cyp19a1a* reverse
7	GTATCAGGGGCTGCAATCAC	*cyp19a1b* forward
8	AGCTTTCGGCAGATAACGTC	*cyp19a1b* reverse
9	GGGCCAAAAGGACAGCTAC	*β-actin* forward
10	CAGGGTCAGGATACCCCTCT	*β-actin* reverse

Table 3. Body length and mass of *Amphiprion ocellaris* kept in groups of three for 10 days. Values are means ± SE and significant differences between social ranks are indicated by different letters. Statistical significance is defined as $P < 0.05$ on the basis of ANOVA and Fisher's PLSD.

	α	β	γ
Length (mm)	49.5 ± 0.96^{a}	45.8 ± 1.12^{ab}	44.4 ± 1.50^{b}
Mass (g)	2.39 ± 0.15^{a}	1.95 ± 0.17^{ab}	1.72 ± 0.15^{b}

Indianapolis, IN, USA). The specific oligonucleotide primer sets used in real-time PCR are listed in Table 2. *β-actin* was used as an internal control to normalize cDNA abundance. The specificity of the primer sets was confirmed by DNA sequencing.

Real-time PCR was performed on a MiniOpticon Real-time PCR System (Bio-Rad, Hercules, CA, USA) with SYBR Green fluorescent labeling. Real-time analysis was performed with a final reaction volume of 25 μL using <100 ng cDNA, 12.5 μL iQTM SYBR Green Super Mix (Bio-Rad), and 10 μM of each primer. The cycling parameters were 95°C for 3 min and then 34 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by a dissociation stage of 72°C for 7 min and 65°C for 1 min.

The cycle threshold (Ct) was calculated automatically with a manual baseline set at 3 to 15 cycles. The acceptability of each triplicate reaction was set at a Ct standard error (SE) of 0.85. Each set of data for social rank was normalized against the data from group-housed control fish (GHC; reference gene, $n = 5$), which did not form social ranks due to the high stocking density. The normalized data were compared by using the equation of Jemiolo and Trappe (2004), which compares changes in the difference between the Ct of the gene of interest and a reference gene, expressed as:

$$\text{fold change} = 2^{-\Delta\Delta Ct}.$$

Statistics

Statistical analyses were performed with StatView + Graphics 5.0J software (Abacus Concepts, Inc., Berkeley, CA, USA; no longer available); $P < 0.05$ was considered to indicate statistical significance. To compare behavioral elements, except for the frequency of lunging at other fish, we used a repeated two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test. Physical parameters, plasma cortisol concentrations, and the frequency of lunging at other fish were compared by one-way ANOVA followed by Fisher's PLSD. Gene transcription levels were compared by one-way ANOVA followed by Dunnett's multiple comparison test.

RESULTS

Physical parameters of fish

ANOVA showed significant differences in body length ($F_{2,11} = 4.089$, $P = 0.047$) and mass ($F_{2,11} = 4.229$, $P = 0.043$); α individuals were significantly longer and heavier than γ ones, and β individuals were intermediate between the two (Table 3).

Behavioral observations

Social rank in groups of three individual *A. ocellaris* formed at the early stage of grouping (i.e., on day 1 of the experiment), and the ranks had not changed by the termination of the experiment. ANOVA revealed a significant (social rank) effect on behavior: $F_{2,12} = 16.017$, $P < 0.001$ for the amount of time spent in the shelter, $F_{2,12} = 16.973$, $P < 0.001$ for lunging, $F_{2,12} = 10.363$, $P = 0.002$ for being the target of lunging, and $F_{2,12} = 8.996$, $P = 0.004$ for trembling. However, there was no significant (time course × social rank) interaction effect. Thus, the behavioral parameters were compared using the total frequency during the entire period of observation.

Post hoc analysis revealed that α individuals spent significantly more time in the shelter than other individuals throughout the experimental period, whereas β and γ individuals rarely entered the shelter (Fig. 1A). Alpha individuals also displayed the most frequent lunging (Fig. 1B), the least trembling (Fig. 1D), and were the least likely to be a target of lunging (Fig. 1C). One-way ANOVA revealed that social rank had a significant effect on the frequency of lunging at other fish ($F_{5,18} = 27.394$, $P < 0.01$, Fig. 1E). Post hoc analysis revealed that α individuals lunged most frequently at β individuals. The frequencies of β fish lunging at α, γ lunging at α, and γ at β were the lowest, and the frequencies of α and β fish lunging at γ were intermediate.

Blood cortisol values

ANOVA revealed a significant difference in blood cortisol values according to social rank ($F_{2,11} = 4.124$, $P = 0.046$), and post hoc analysis showed that α individuals had significantly higher blood cortisol than β and γ individuals, between which there was no significant difference (Fig. 1F).

Gene analysis

Specific primers were designed for real-time PCR, and gene transcription in the brains of fish of each social rank was compared (Fig. 2). ANOVA revealed significant differ-

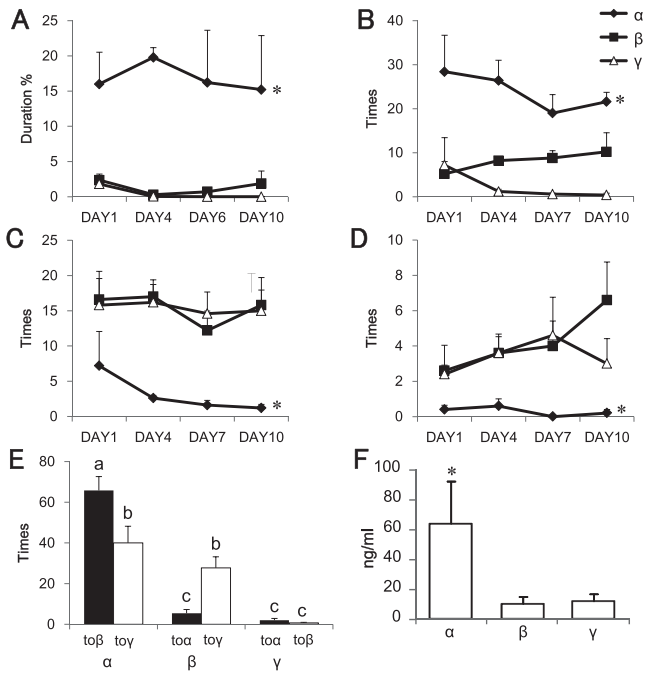

Fig. 1. Behavioral parameters and blood cortisol values of individual α, β, and γ false clown anemonefish *Amphiprion ocellaris* kept together for 10 d. **(A)** Proportion of time spent in the shelter by individuals of each social level; **(B)** total frequency of lunging behavior; **(C)** frequency of lunging at other members of the social group; **(D)** total frequency of being the target of lunging; **(E)** frequency of trembling; and **(F)** blood cortisol values. Values are means ± SE; *, $P < 0.05$; different letters above bars indicate statistically significant differences (Fisher's PLSD).

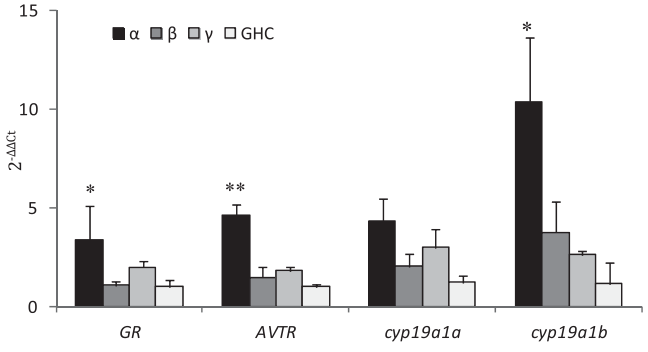

Fig. 2. Transcription of genes encoding for glucocorticoid receptor (*GR*), arginine vasotocin receptor (*AVTR*), brain aromatase (*cyp19a1b*), and gonad aromatase (*cyp19a1a*) in the brains of α, β, and γ *Amphiprion ocellaris* kept together for 10 d. Values are means ± SE. *, $P < 0.05$; **, $P < 0.01$, compared with a reference gene (i.e., value in group-housed control, GHC) (Dunnett's multiple comparison test).

ences among social ranks in the stress-related genes *GR* ($F_{3,15} = 3.800$, $P = 0.039$) and *AVTR* ($F_{3,15} = 14.415$, $P < 0.001$). Post hoc analysis revealed the highest transcription of *GR* and *AVTR* in α individuals.

ANOVA indicated that there were significant differences in the transcription of *cyp19a1b* genes among social ranks ($F_{3,15} = 5.004$, $P = 0.018$), and post hoc analysis showed higher transcription of *cyp19a1b* in α individuals than in the other social ranks. Although there was a trend toward higher transcription of *cyp19a1a* in α individuals, ANOVA showed no significant differences between social ranks ($F_{3,15} = 2.746$, $P = 0.089$).

DISCUSSION

Our results showed that social rank in a group of three individual *A. ocellaris* formed at the early stage of grouping. On day 10 of the experiment, α individuals on average had a greater body mass than those of other social ranks. A previous study by our group showed that differences in body mass among social ranks occur because individuals of higher social rank hinder those of lower rank from feeding (Iwata et al., 2008), and it has also been reported that subordinate fish can adjust their growth strategically to avoid conflict among group members (Buston, 2003; Heg et al., 2004). However, the experimental period in the present study was short, and there is no evidence that the differences in body mass resulted only from differences in food intake. Body size is an important determinant of dominance relationships (Abbot et al., 1985; Beacham, 1987); therefore it seems likely that social rank was established by slight differences in body mass at the beginning of the experiment.

The social rank of each individual was clearly distinguishable by observing the frequency of lunging and being targeted by lunging; α individuals were lunged at least and lunged the most, displaying fierce aggressiveness, especially toward β individuals that tried to enter the shelter. β individuals lunged mostly at γ fish, and it was difficult for γ fish to get near the shelter because of the attacks by both α and β individuals. The α individuals seemed to monopolize the inside of the shelter, and their blood cortisol levels were higher than those of the other group members.

It had been thought that social suppression from dominant individuals could elevate blood cortisol levels and suppress reproductive activity in individuals with subordinate social rank (Blanchard et al., 1993). But recently it was found that for some simian species, the social rank having higher blood cortisol values may vary with the species, which is a reflection of variations in social structures (Abbott et al., 2003). For example, male dominance rank in wild chimpanzees *Pan troglodytes* correlated positively with urinary cortisol excretion in a stable dominance hierarchy, and cortisol excretion also correlated positively with rates of male aggression (Muller and Wrangham, 2004). Similarly, high-ranking male Japanese macaques *Macaca fuscata* secreted significantly higher levels of cortisol than low-ranking males (Barrett et al., 2002). These results suggest that there may be costs associated with dominance. Furthermore, recent studies revealed that dominant individuals of cooperative breeders, including the teleost species *Neolamprologus pulcher* (African cichlid), tended to show elevated glucocorticoids more often than subordinates (Creel, 2001; Mileva et al., 2009). Anemonefishes are not categorized as cooperative breeders, but they are highly social and have monogamous mating strategies; the male mainly cares for eggs and guards the nest (Moyer and Bell, 1976; Buston, 2004). High cortisol values in dominant *A. ocellaris* in the present study may reflect such a social structure in anemonefishes.

In contrast, in another study of olive baboons *Papio anubis*, a male being challenged for his more dominant position tended to display higher basal cortisol concentrations (Sapolsky, 1992). In the nonmammalian vertebrate lizard *Anolis carolinensis*, chronically elevated plasma glucocorticoids reliably inhibit aggressive behavior, but acute elevation of plasma glucocorticoids may either promote an actively aggressive response or be permissive to escalated aggression or activity (Summers et al., 2005). The higher blood cortisol in dominant *A. ocellaris* compared to other members of the group in the present study might be due to the dominant individual's struggle to establish dominant status at the early stage of social rank formation of the group.

Stress-related genes in the brain (i.e., *GR* and *AVTR*), the transcription of which is associated with blood cortisol values, also showed higher transcription in α individuals compared to other group members. As in other vertebrates, upregulation of *GR* mRNA in teleost fish is induced by an acute stress response as a result of elevation of blood cortisol values (Acerete et al., 2007). In the green molly *Poecilia latipinna*, arginine vasotocin (AVT) neurons innervate the corticotroph cells of the pituitary (Batten et al., 1990) and influence adrenocorticotropin, and thus cortisol secretion, in the rainbow trout *Oncorhynchus mykiss* (Baker et al., 1996). In socially controlled protogynous bluehead wrasse *Thalassoma bifasciatum*, aromatase-immunoreactive fibers are closely associated with AVT-immunoreactive neurons in the preoptic area, indicating the interaction between local estrogen synthesis and signaling systems that subserve social behavior (Marsh et al., 2006). Moreover, our previous studies revealed that brain AVT neurons were modulated by social rank formation in *A. ocellaris* (Iwata et al., 2010a; Iwata et al., 2010b). Taken together, these findings suggest that the α individuals in the current experiment were acutely stressed.

In the brains of ambisexual *A. ocellaris,* transcription of the *cyp19a1b* gene, which encodes brain-type P450 aromatase, was higher in α individuals than in other social ranks. There was also a trend of higher *cyp19a1a* levels, which encodes gonad-type P450 aromatase, in α individuals. During the social rank formation period, the dominant individual is in a stressful situation and behaves aggressively toward other group members, possibly because of the need to establish and maintain its dominant status. This stressful social situation may influence aromatase activity, possibly via the hypothalamo–pituitary–adrenal axis.

It is well known that gonochoristic teleost species with TSD demonstrate changes in sex ratio in response to changes in environmental temperature. For example, high temperatures during the sex differentiation period in Japanese flounder produce a male-dominant population because *cyp19a1a* transcription is suppressed (Kitano et al., 1999; Kitano et al., 2001). Cortisol treatment mimics the effects of high temperature in inducing masculinization in larval-stage pejerrey; this induction is associated with the suppression of *cyp19a1a* transcription (Hattori et al., 2009). In Japanese flounder, the cAMP-responsive element (CRE) in the *cyp19a1a* promoter region reportedly binds to the cortisol–GR complex and suppresses gene transcription (Yamaguchi et al., 2010). Moreover, in sex-changing fish, sex-changing dominant females of the protogynous blue-banded goby *Lythpnus dalli*, which displays an increased number of aggressive acts, have lower brain aromatase activity (Black et al., 2005).

In contrast, *cyp19a1a* and *cyp19a1b* in the brain of *A. ocellaris* in the current experiment seemed to be upregulated by the elevation of blood cortisol. However, in several TSD teleost species, such as those of the genus *Sevastes*, high temperature induces the development of a female-dominant rather than a male-dominant population (Omoto et al., 2010). Furthermore, high blood cortisol values are detected in male-to-female sex-changing *A. melanopus* 20 d after female removal (Godwin, 1994). It was also reported that polymorphisms in the promoter region of *cyp19a1a* of Japanese flounder are associated with blood E_2 level (He et al., 2009). These results suggest the existence of a different regulatory mechanism that leads to feminization in certain stress situations.

It seem improbable at present that elevation of aromatase gene transcription in the brain is directly linked to sex determination in α individuals, as sex determination in ambisexual fish takes several months (Hattori and Yanagisawa, 1991; Iwata et al., 2008; Iwata et al., 2010a). Moreover, persistently elevated cortisol levels would downregulate *GR*, which would in turn reduce blood cortisol values (Pottinger, 1990; Maule and Schreck, 1991). A previous study by our group revealed that after six months in group living, blood cortisol values tended to be higher in β individuals of *A. ocellaris*, which displayed submissive behavior (i.e., trembling) more frequently than α or γ individuals (Iwata et al., 2008). These results suggest that the social stress levels of each individual change over time; thus, more research is needed to reveal the relationship between endocrine changes associated with changes in social interactions in a group and sex determination mechanisms of *A. ocellaris*. However, it appears that aromatase genes may play a role in the brain during sex differentiation in *A. ocellaris* because social rank, which in turn leads to gonadal sex differentiation, is determined at the very early stages of social group formation.

Here, we have demonstrated for the first time that the transcription of P450 aromatase genes in the brain of *A. ocellaris* is upregulated by social stress. However, at present it is unclear whether cortisol directly affects gene transcription. Thus, further experiments, for example evaluation of gene transcription in fish subjected to cortisol administration, will be needed. Further examination of the time course of gene transcription changes in the gonad and in the brain as well as the analysis of upstream regions of these genes may reveal the mechanisms of sex differentiation or sex change in protandrous anemonefish.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research (no. 22570069) from the Japan Society for the Promotion of Science. The authors thank Dr. T. Furuta of the Central Research Institute of Electric Power Industry for providing the anemonefish used in this study. We also thank Dr. M. Ichikawa of the Tokyo Metropolitan Institution of Neuroscience and Dr. M. Yokosuka of Nippon Veterinary and Life Science University for their valuable advice.

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(Received January 10, 2012 / Accepted June 13, 2012)