Hormones and Behavior 103 (2018) 62–70

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Dynamic regulation of brain aromatase and isotocin receptor gene expression depends on parenting status

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ARTICLE INFO

Keywords:
Parental care
Paternal care
Fathering
Isotocin
Arginine vasotocin
Arginine vasopressin
Oxytocin
Receptors
Estradiol
Estrogen
11-ketotestosterone, testosterone
Aromatase
Anemonefish
Amphiprion ocellaris

ABSTRACT

Fathering behavior is critical for offspring survival in many species across diverse taxa, but our understanding of the neuroendocrine mechanisms regulating paternal care is limited in part because of the few primarily paternal species among the common animal models. However, many teleosts display primarily paternal care, and among the teleosts, anemonefish species are particularly well suited for isolating molecular mechanisms of fathering as they perform parental care in isolation of many other typically competing behaviors such as territorial defense and nest building. The goal of this study was to determine the extent to which whole brain gene expression levels of isotocin receptors, arginine vasotocin receptors, and aromatase as well as circulating levels of the bioactive sex steroid hormones estradiol (E2) and 11-ketotestosterone (11KT) vary in association with parenting behavior in *Amphiprion ocellaris*. Brain aromatase and IT receptor gene expression were higher in both males and females that were parenting versus not. IT receptor expression was overall higher in males than females, which we interpret is a reflection of the greater parental effort that males display. Aromatase was overall higher in females than males, which we conclude is related to the higher circulating E2, which crosses into the brain and increases aromatase transcription. Results suggest both aromatase and IT receptors are dynamically upregulated in the brains of *A. ocellaris* males and females to support high levels of parental effort.

1. Introduction

Given the diverse and environmentally dependent nature of sociality, individuals are often required to make rapid and dynamic behavioral shifts within their social groups in a sex specific manner. As social circumstances and group dynamics vary, individuals must find ways to modify their behavior in order to maximize their fitness within the social structure (Lema et al., 2015; O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012). Understanding how species and sex specific behaviors are coordinated among, and within individuals is of keen interest in social neuroscience, especially as behavioral variation corresponds to variation within the brain (Goodson, 2008; Lee et al., 2009; Okhovat et al., 2015; Olazabal and Young, 2006). One such example of a stark behavioral shift is parental care. In contrast to maternal care, where pregnancy or gravidity induce a suite of physiological and hormonal changes prior to parturition or egg laying, physiological cues to fathers are more subtle. Notwithstanding the physiological subtleties priming fathers to care, fatherhood is often critical for offspring survival. Of course human males father, and this behavior is displayed in a highly variable way. Consequently, fatherhood has recently become a highly socially relevant topic (Bales and Saltzman, 2016; Saltzman and Ziegler, 2014).

One obstacle that has limited our understanding of fathering behavior is the paucity of primarily paternal species among the common animal models. In mammals, male care is rare, and when it does occur, is often accompanied by active courtship and nest building. These preceding behaviors may modify the brain prior to parental care (Dulac et al., 2014; Wang et al., 1999; Young et al., 2005). This combination makes disentanglement of the underlying neural substrates for parental behavior challenging. Compared to mammals, male care is more common among teleost fishes. Recent findings in fish models have broadened our understanding of the neuroendocrine regulation of fathering behavior in vertebrates by showing that paternal care shares certain features in common with maternal care, such as the prominent role of isotocin signaling. Conversely, novel features such as the role of steroidogenesis within the brain have also been discovered (Deangelis et al., 2017; Deangelis and Rhodes, 2016; Kleszczyńska et al., 2012; Knapp et al., 1999; Kulczykowska and Kleszczyńska, 2014; O'Connell et al., 2012; Pradhan et al., 2014; Rodgers et al., 2006).

The nonapeptides arginine vasopressin (AVP) and oxytocin (OT), as

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https://doi.org/10.1016/j.yhbeh.2018.06.006
Received 10 March 2018; Received in revised form 11 June 2018; Accepted 15 June 2018

well as their non-mammalian homologs arginine vasotocin (AVT) and isotocin (IT), have been implicated as key neuromodulators in a variety of social behaviors, including parental care (Bales et al., 2004; Deangelis et al., 2017; Feldman et al., 2010; Gubernick et al., 1995; Insel and Young, 2000; Kozorovitskiy et al., 2006; Kulczykowska and Kleszczyńska, 2014). AVP/AVT and OT/IT neuron cell bodies reside primarily within the preoptic area of the hypothalamus (POA), and project widely throughout the brain (Goodson, 2008; O'Connell et al., 2012; Olazábal et al., 2013; Rosenblatt and Ceus, 1998). Furthermore, these nonapeptide signaling processes have often been cited as functioning in a sex specific manner. For example, the AVP signaling system underlies pair bonding more prominently in male prairie voles, while in female voles OT signaling pathways play a more important role (Young and Wang, 2004). Hence it is possible, if not likely, that these systems operate differentially in males and females with respect to parental care.

Cumulative evidence suggests that the nonapeptide neurons themselves are modulated, in part by sex steroid hormones, which bind to nuclear receptors and thereby alter gene expression. For example, in the California mouse, local action of brain aromatase, which converts testosterone (T) into estradiol (E2), is critical for high levels of paternal care (Trainor and Marler, 2001, 2002). Teleost fishes have higher brain aromatase activity than any other vertebrate group which may facilitate high levels of fathering behavior in this taxon (Forlano et al., 2001). One important hypothesized function of elevated brain E2 is upregulation of nonapeptide signaling via interactions with E2 nuclear receptors within nonapeptide neurons. E2-nuclear receptor interactions can alter gene transcription in nonapeptide neurons in a way that augments their function (Gimpl and Fahrenholz, 2001). In addition to regulating the synthesis of the nonapeptides within POA neurons, E2 facilitates the production of OT receptors throughout the brain in rats (Tribollet et al., 1990), and increases OT receptor binding in the preoptic area and lateral septum at the onset of parenthood in female rodents (Champagne et al., 2001). Taken together these findings suggest that local conversion of T to E2 within the brain, or E2 from circulation that crosses into the brain play an important role in OT signaling, and in the regulation of parental care. However, whether the function of brain aromatase activity is conserved independent of sex and across species in the regulation of parental care remains unclear.

Compared to the functional role of E2 in the brain, the role of the bioactive androgens in regulating nonapeptide neurons and signaling systems involved in fathering behavior is far less understood. In the blue banded goby, conversion of T to 11KT within the brain is critical for high levels of male parental care (Pradhan et al., 2014), and in the bluegill sunfish, blockade of androgen receptors decreases nest defense (Dey et al., 2010). However, we have previously shown that circulating 11KT did not vary across the breeding cycle in male anemonefish, suggesting a minimal or non-existent role of circulating 11KT in fathering (Deangelis and Rhodes, 2016). So while some minimum level of androgens may be necessary for males to display paternal care, additional molecular substrates are likely responsible for the shift from non-parental to parental behavior. Taken together, the current literature suggests fathering behavior is mediated by increased OT/IT signaling via E2 aromatized from T locally within the brain, with minimal contributions from circulating bioactive sex steroids. However, the extent to which aromatase and IT/OT receptor gene expression are dynamically upregulated in male brains as they transition from inactive to active fathers remains unknown. Moreover, how these signaling pathways are either conserved across sexes, or function in a sex specific manner remains unclear.

In contrast to OT, the functional role of AVP/AVT signaling in the regulation of parental care is less understood. However, some recent studies have shown AVP as an important regulator of fathering behavior, nest defense, and territoriality. In prairie voles, AVP injections into the lateral septum, a brain region known to be involved in mediating behavioral acts related to offspring care, enhanced paternal care (Dulac et al., 2014; Wang et al., 1994), and in the California mouse, AVP mRNA levels in the paraventricular hypothalamic nucleus correlated positively with the number of paternal behaviors displayed (De Jong et al., 2012). Similarly in pipefishes, AVT peptide concentrations are higher in the brains of parenting males vs. non-parenting males (Ripley and Foran, 2010). And in sticklebacks AVT peptide concentrations in the whole brain were highest in aggressive males actively caring for eggs (Kleszczyńska et al., 2012). While these studies suggest AVP/AVT signaling is important in paternal care, the specificity of the relation to offspring care as compared to involvement in aggression and territorial defense is less clear, as multiple behaviors occurred simultaneously in these animal models.

Among teleosts, the anemonefish *Amphiprion ocellaris* presents an exciting opportunity for exploring neuroendocrine regulation of male parental care. *A. ocellaris* lives in relatively small and simple social groups, where pair bonds and social hierarchies are established long before mating occurs. Therefore, there is no active courtship, nest building or intraspecific aggressive interactions co-occurring during high levels of parental care, enabling the underlying regulatory mechanisms to be more specifically extricated (Deangelis et al., 2017; Deangelis and Rhodes, 2016; Iwata et al., 2012; Iwata et al., 2010). Additionally, comparing brains of males and females that are actively parenting to those, which are not actively parenting, allows isolation of the molecular mechanisms regulating parental care.

We have recently shown that in *A. ocellaris,* blockade of IT receptors significantly reduces paternal care, whereas blockade of AVT V1a receptors increases fathering behavior (Deangelis et al., 2017). In a previous report we also documented that circulating E2 varies over the breeding cycle in females, but not males (Deangelis and Rhodes, 2016). Therefore, we hypothesized that circulating sex steroids (at least E2 in females), brain aromatase, AVT receptor V1a, and IT receptor gene expression would dynamically change as individuals shift in status between parenting and non-parenting. More specifically, we hypothesized that brain aromatase gene expression levels would increase in response to parental care in males, but still display relatively higher levels in females, due to positive feedback regulation by circulating E2 (Callard et al., 2001; Menuet et al., 2005). Further, we hypothesized that IT receptor gene expression would be higher in individuals actively caring for eggs compared to non-active parents. We expected that IT receptor gene expression would be higher in males than females, as a reflection of their higher levels of parental behavior. Finally, we hypothesized that AVT V1a receptor gene expression would show the opposite pattern, lower in parenting individuals and lower in males compared to females, based on the pharmacology result where blockade of V1a increased parenting in males. The goal of this study was to determine the extent to which brain aromatase, AVT V1a receptor, and IT receptor gene expression vary within the context of parenting and in relation to circulating bioactive sex steroid hormone levels, E2 and 11KT.

2. Materials and methods

2.1. Animals and husbandry

All fish used were offspring bred in house from broodstock obtained from ORA (Oceans Reefs and Aquariums, Fort Pierce, FL). Fish were kept in 20-gallon tall aquariums (24″ × 12″ × 16″) integrated via plumbing to a large circulating filtration system. Conditions were set to mimic the natural environment with a pH between 8.0 and 8.4, temperature range of 79–82 °F, photoperiod of 12:12 (lights on at 7:00 am and off at 7:00 pm), and specific gravity of 1.026. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Fish were kept in either one of two social housing conditions. First, animals were kept in pairs, containing one dominant female and one subordinate male. Each tank containing one 6″ terra-cotta pot, serving

as a surrogate host anemone and nesting site. All fish in this social setup had been established as pairs for over 2 years prior to experimental observations. Secondly, non-reproductive male anemonefish were housed in groups of over 30 individuals without a terra cotta pot. This housing condition suppresses sexual maturation and inhibits any social structure from forming.

A total of 22 males, 22 females, and 8 undifferentiated group-housed fish were used in this study. The females were on average 7.0 cm (± 0.74 SD, range 5.7–8.5 cm) long and had a body mass of 7.6 g (± 0.91 SD, range 3.75–13.80 g). The males had a mean body length of 5.5 cm (± 0.39 SD, range 4.8–6.3 cm) and mass of 3.4 g (± 0.88 SD, range 2.23–4.81 g). Finally, the undifferentiated group-housed individuals had a mean body length of 5.3 cm (± 0.31 SD, range 4.9–5.9 cm) and mass of 3.48 g (± 0.65 SD, range 2.83–4.55 g). There were no differences in weight or length between males and females in the treatment groups described below.

2.2. Experimental design

All mature pairs were monitored carefully for a 3-month period and observed spawning events recorded. Pairs that were spawning regularly (inter-spawn intervals of fewer than 21 days) were deemed 'Active' spawners ($n = 11$). Pairs were deemed 'Non-Active', if they had not spawned during the 3-month observational time period (n = 11). Pairs displaying intermittent spawning events, a spawn period > 21 days were not used in this study. Following the 3-month observation period, and only for actively spawning pairs, parental behaviors were quantified for 7 consecutive days following a spawning event. Additionally, the number of eggs on the day the eggs were laid, and the number of eggs 7 days after the eggs were laid were also quantified. Males and females from actively spawning pairs were then euthanized on day 7, following behavioral analysis and egg counts. No behaviors were quantified for non-active pairs (as no parental care occurred). Additionally, individuals from group-housed conditions ($n = 8$) were also sampled for comparison.

2.3. Behavioral quantification

For active pairs, all parental behavioral displays by both males and females were quantified from video recordings taken between 14:00 and 15:00 h each day that eggs were present in the nest (7 days). Video recordings were taken for 15 min and scored following a 5-minute acclimation period (time provided for fish to display normal behavior following camera setup in front of their home aquarium) for a total of 10 min. Video recordings were scored using Jwatcher Behavioral Event Recording Software (Blumstein and Daniel, 2007). The total number of nips (mouthing of the eggs to keep them clean of fungus and debris) and fans (using pectoral and caudal fins to provide oxygen rich water to the eggs) were scored for each day. The time spent in the nest (proportion of the 10-minute video that individuals were in the terra cotta pot) was also scored. The total number of behaviors and time in the nest was averaged per day for statistical analysis.

2.4. Blood sample collection

All blood sampling occurred between 14:00 and 15:00 h in order to control for any diurnal changes in hormone levels. Fish were placed between two wetted paper towels with only the caudal region exposed. Blood was then sampled from the lateral caudal vein using a 27-gauge heparinized butterfly needle (Terumo Medical Products) mounted on a 1 ml syringe (BD Syringe). Between 50 µl to 250 µl of blood was collected from each individual. Sample volumes varied due to individual variation in body size, and proficiency of the procedure. All blood samples were collected within 2 min of removal from the home aquarium (a time previously validated as having no effect on measured hormone concentrations (Deangelis and Rhodes, 2016)). Immediately following collection, blood was dispelled into 0.6 ml centrifuge tubes and spun in a chilled centrifuge (4 °C, Eppendorf Centrifuge 5417R) at 4000 rpm for 15 min. The plasma supernatant was then extracted, aliquoted in 20 µl samples, and stored at −80 °C until assayed.

2.5. Hormonal measurements

Plasma was assayed using previously validated commercially available enzyme immunoassay kits for E2 (Calbiotech, Lot NO. ESG4324, range of 3–300 pg/ml) and 11KT (Cayman Chemical, Item No. 582751, range 0.78–100 pg/ml). Each plasma sample was diluted 1:30 in assay buffer as done in Deangelis et al. (2017), prior to analysis following kit instructions. All samples were run in duplicate in a single assay. Subsequent absorbance was read using the Epoch Microplate Spectrophotometer (BioTek Instruments).

2.6. RNA extractions and cDNA synthesis

The whole brain (excluding the spinal cord and olfactory bulbs) of each fish was dissected out within 3.5 min of removal from their home tank, placed in RNAlater Stabilization Solution (Thermo Fisher, Cat. No. AM7020) at a volume 5 times the volume of the brain, and stored at 4 °C overnight. Brains were then transferred to clean microcentrifuge tubes and stored at −80 °C until RNA extractions were performed (no longer than 1 month). Whole brain RNA extractions were performed using the RNeasy Lipid Tissue Mini Kit (Qiagen, Cat. No. 74804) following manufacture instructions. RNA was extracted from whole brains to identify the main effects of parenting on gene expression independent of brain region specific expression patterns. RNA concentrations were then quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). RNA quality number (RQN) and 28S/18S ratios were determined using a Fragment Analyzer Automated CE System (Advanced Analytical AATI, Table 1) at a concentration of 100 ng/µl. With remaining aliquots, RNA concentrations were normalized to 500 ng/µl prior to reverse transcription. RNA was reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Cat. No. 1708890) following manufacture's instructions, in a total volume of 20 µl per reaction. To control for potential genomic DNA contamination, samples were treated with RNase free DNase I (New England BioLabs, Cat. No. M0303S) and negative control reactions were run without the iScript transcriptase.

2.7. Primer validation

Primers were designed to recognize mRNA sequences of vasotocin receptor 1A (*V1ar*) (GenBank #AB669615.1), isotocin receptor (GenBank #AB669618.1), brain aromatase (*cyp19a1b*) (Genbank #AB918722.1), and beta actin (GenBank #AB9212201.1) using Primer-BLAST software (NCBI) against sequences specific to *A. ocellaris*. All of the genes measured in this study have been previously sequenced and submitted to GenBank (Iwata et al., 2012; Iwata et al., 2010). The V1ar, IT receptor, and beta actin genes may have additional paralogs as a consequence of a genome duplication in the evolutionary history of teleost fishes, and hence it is important to note that for these genes, only one paralog was quantified and the specificity of which paralog was assayed is unclear. Primer pair specificity was blasted against the reference genome of the damselfish *Stegastes partitus* (taxid: 144197), the closest relative of *A. ocellaris*, where the full genome is available. Primer melting temperatures were set with a minimum of 58 °C, a maximum of 61 °C, and an optimum of 60 °C. The maximum number of primers to screen was set at 1000, with primer sizes set between 18 and 27 base pairs, with an optimum at 24 base pairs, and a max Poly-X at 3. Primers were then analyzed for hairpins, and primer dimers using OligoAnlayzer (IDT Technologies) prior to serial dilution validations. Only primers showing hairpin analysis with ΔG (kcal mol^{-1}) values < 2, and primer-dimer values of $\Delta G < 6$ were tested in serial

Table 1
RNA Quality Table shows the RNA quality number (RQN) as given by the AATI Fragment Analyzer as a measure of RNA integrity. RNA concentrations, 28s:18s ratio, 260:280 ratio, and 260:230 ratio are also given as an index of RNA integrity.

FISH ID	RQN	28S:18S Ratio	RNA concentration in ng/μl	260:280 Ratio	260:230 Ratio
10j	8.8	1.5	322.4	2.1	2.2
1j	8.7	1.6	358.8	2.09	2.18
2j	8.8	1.6	381.3	2.09	1.85
4j	8.9	1.6	404.6	2.1	2.07
5j	8.9	1.6	193.1	2.11	2.05
6j	8.9	1.4	274.8	2.12	2.23
8j	8.9	1.7	165.9	2.1	1.85
9j	8.5	1.4	140.5	2.09	2.14
a11f	8.9	1.7	489.3	2.07	2.13
a11m	9.1	1.7	403.3	2.11	2.24
a12f	9.1	1.7	445.4	2.11	2.09
a12j	9.2	1.6	234.1	2.09	2.08
a12m	9.3	1.7	226.8	2.1	2.17
a13f	8.6	1.4	441.9	2.09	2.12
a23f	9.1	1.7	375.2	2.1	2.16
a23m	8.9	1.7	371.9	2.11	2.15
a25f	8.8	1.7	486.1	2.03	1.97
a25j	8.9	1.7	384.8	2.11	2.21
a25m	8.8	1.7	432.7	2.1	2.2
b11f	9.5	1.8	342.3	2.11	2.21
b11j	9.6	1.8	280.6	2.09	2.2
b11m	9.2	1.7	246	2.09	2.14
b14f	9.2	1.9	458.1	2.1	2.27
b14m	9.4	1.8	324.5	2.13	2.2
b23f	9.1	1.9	351.9	2.11	2.31
b23m	9.1	1.8	414.2	2.11	2.2
b25f	9.6	2.1	486.7	2.1	2.24
b25m	9.1	1.7	215.7	2.11	2.13
c12f	8.7	1.7	357	2.1	2.24
c12m	9.2	1.7	346.1	2.1	2.13
c14f	9.1	1.9	379.6	2.11	2.19
c14j	9.2	1.7	274.5	2.1	1.95
c14m	8.8	1.6	428.4	2.11	2.08
c21f	9.5	1.6	472.5	2.1	2.16
c21j	9.2	1.6	154.9	2.08	2.05
c21m	9.1	1.5	434.4	2.11	2.15
c25f	9.1	1.8	369.6	2.11	2.23
c25m	8.6	1.4	339.9	2.12	2.07
t11f	9.4	1.8	504.8	2.09	2.31
t11 m	9.5	1.7	264.5	2.09	2.25
t12f	9.2	2	420	2.12	1.75
t12j	9.4	1.9	294.1	2.11	1.97
t13f	9.1	1.5	401.8	2.09	2.33
t13 m	9.4	1.8	420.2	2.11	2.09
t14f	9.2	1.5	447	2.1	1.46
t14j	9	1.7	352.6	2.12	2.12
t14 m	9.3	1.9	250.7	2.12	1.65
t15f	9.5	1.9	417.4	2.1	2.25
t15 m	9.4	1.7	368.6	2.12	2.19
t20f	9.1	1.7	384	2.09	2.27
t20 m	8.5	1.6	461.5	2.1	2.18
t2 m	9	1.7	329	2.11	2.19
t6f	9.3	1.8	414.1	2.09	2.22
t7 m	9.3	1.9	357.2	2.13	2.12

dilutions. All primers were commercially purchased from IDT technologies (Coralville, IA). Following cDNA synthesis, samples were diluted in a 4-fold series and checked for optimal efficiencies and a single melt curve. All primers displayed an efficiency between 90 and 110% and an R^2 value of over 0.96 (Table 2).

2.8. Relative gene expression quantification

Gene expression was quantified using the SSo Advanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories, Cat. No. 1725271) and CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Cat. No.1855201). All reactions were run in triplicate. The cycle parameters were 98 °C for 30 s, 95 °C for 10 s, 52–55 °C (depending on primer pair, see Table 2) for 30 s, then plate read and repeated for 40 cycles. Following the 40 cycles, a melt curve was performed with an increase in temperature from 58 °C to 95 °C at 0.5 °C increments, followed by a final plate read.

The cycle threshold (Ct) was calculated automatically in the CFX Connect™ software. Only triplicate reactions with a Ct standard error (SE) under 0.85 were used in statistical analysis. Gene expression data were then computed 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 (beta actin), expressed as:

Fold change = $2^{-\Delta\Delta Ct}$ (Jemiolo and Trappe, 2004). Beta actin was chosen as a reference gene due to its consistent Ct values across groups, and because it has previously been validated and used as a reference gene in *A. ocellaris* (Iwata et al., 2012).

2.9. Statistical methods

Data were analyzed using R (version 3.3.0 'Supposedly Educational') statistical software (Team, 2013). $P < 0.05$ was considered statistically significant. To determine effects of parental status and sex on gene expression, and circulating hormone levels, a two-way analysis of variance (ANOVA) was performed with sex and status as factors. Effects sizes for these models are reported as eta^2. For males, because of the additional treatment group of 'group housed', a one-way ANOVA was performed. Post-hoc pair wise comparisons of means for both one-way and two-way ANOVAs were performed using Fisher protected least squared differences (LSD) tests. All variances of residuals in these models were checked for normality visually, and using Shapiro-Wilks normality tests ($P > 0.10$, in all comparisons). Data were either log-transformed to meet normality assumptions, or when data could not be normalized, a non-parametric Kruskall-Wallis test was performed. All specific data transformations are noted in the results section. In examining correlations between measured variables, Pearson's product-moment correlation coefficients are reported.

3. Results

3.1. Parental behavior

During the 10-minute video scoring period, males displayed an average of 81 (± 5.91 SEM) total parental behaviors (sum of nips and fans) and spent 77% (± 0.21 SEM) of their time in the nest. Conversely, females displayed an average of 21 (± 3.30 SEM) total parental behaviors and spent only 21% (± 0.03 SEM) of their time in the nest. Both average number of behaviors [$t_8 = -4.63$, $P = 0.0009$] and time spent in the nest [$t_8 = 12.61$, $P < 0.0001$] were significantly different

Table 2
Specific base pair sequences are given for each target sequence measured using Real-Time PCR. Melt temperatures specific to each primer pair are also indicated.

Target sequence	Forward primer	Reverse primer	Melt temperature	Effciency	R-squared
Aromatase (brain)	CAGCGAGCAACTACTACAACAA	ACATGGTACACCGCAGAC	54.5 °C	108.1	0.981
Beta actin	GTTGGTGATGAAGCCCAGAG	ATCTTCTCCATGTCATCCCAGT	55 °C	100.6	0.997
Isotocin receptor	GGAGCATCACACAGACTTCC	GGCCACATGTATGTATCTGAAGG	54.5 °C	104.2	0.992
Vasotocin V1a receptor	TGCCAGCTCATATTAACTTTGGA	TCTCTTCACTGTTTCATGTGTTT	52.0 °C	106.6	0.986

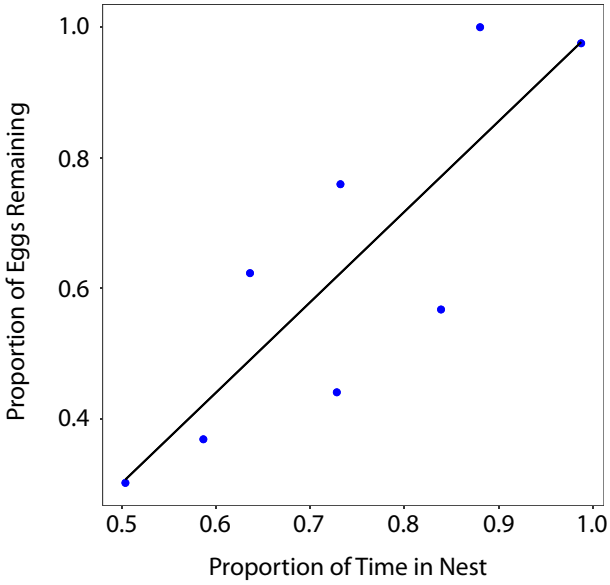


Fig. 1. Male effort pays off in terms of egg survival. The x-axis shows proportion of time males spent in the nest during the 10-minute behavioral quantification period. On the y-axis is the proportion of eggs remaining as calculated by counting eggs from photographs taken on the day of spawning, and 7 days after egg deposition. The two variables are significantly correlated ($r_8 = 0.760$, $P = 0.011$).

between males and females. Additionally, consistent with previous findings, the proportion of time males spent in the nest, and total number of male behaviors, were positively correlated [$r_8 = 0.88$, $P = 0.01$], and the proportion of time males spent in the nest was positively correlated with the proportion of eggs remaining on day 7 [Fig. 1; $r_8 = 0.76$, $P = 0.011$].

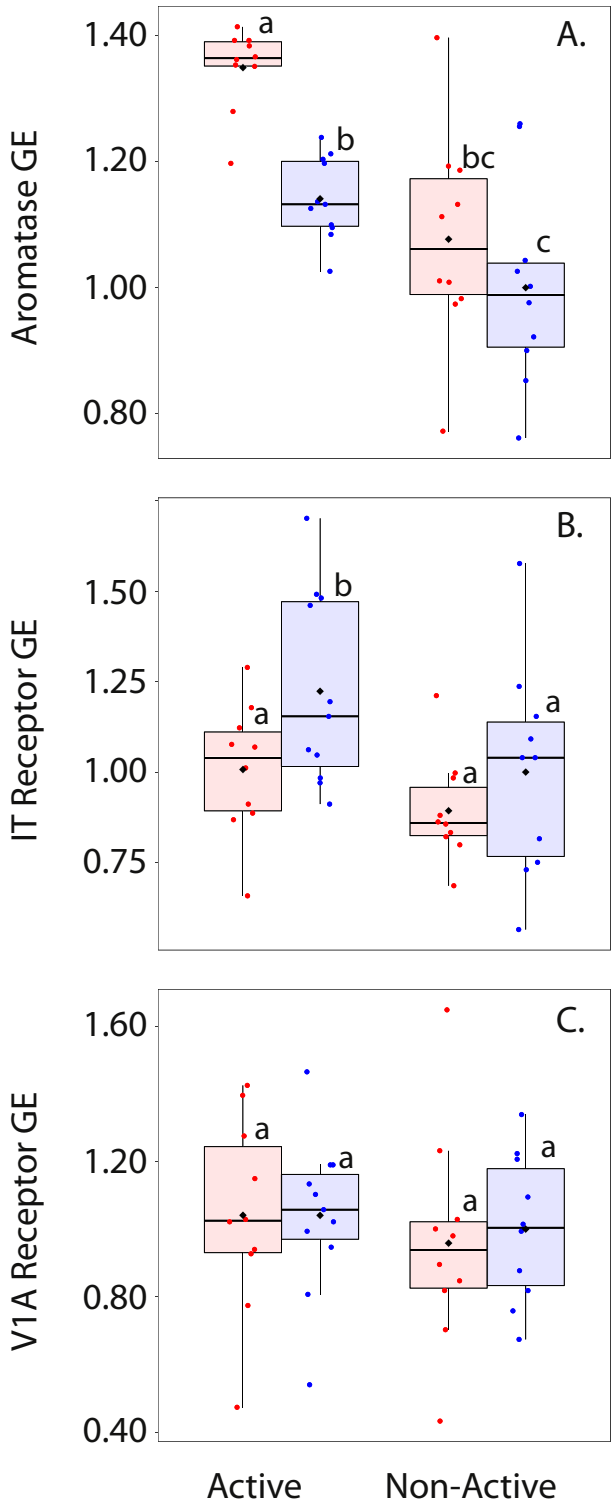
3.2. Brain aromatase gene expression

Values of aromatase gene expression were log transformed in order to meet Shapiro-Wilks normality criterion. Results from the two-way ANOVA showed a significant effect of parenting [$eta^2 = 0.43$, $F_{1,37} = 28.27$, $P < 0.001$] and sex [$eta^2 = 0.27$, $F_{1,37} = 28.27$, $P < 0.001$] on relative aromatase gene expression (Fig. 2A). No significant interaction between sex and parenting was present [$eta^2 = 0.73$, $F_{1,37} = 2.88$, $P = 0.09$]. Overall, females displayed 13% higher aromatase gene expression than males, and individuals that were parenting displayed 20% higher aromatase gene expression than those not actively caring for eggs. Separate analyses by sex showed that females that were actively parenting had 25% higher aromatase gene expression than non-parenting females [$P < 0.001$]. Additionally, males that were actively parenting displayed 8% higher levels of relative aromatase gene expression than those that were not parenting [$P = 0.01$] (Fig. 2C).

Results from the one-way ANOVA comparing males across treatments, including group-housed males, showed a significant effect of treatment on relative aromatase gene expression [$eta^2 = 0.54$, $F_{2,26} = 15.86$, $P < 0.0001$]. Post hoc analyses revealed that actively parenting males showed 14% higher levels of relative aromatase gene expression than non-parenting males [$P = 0.02$], and 41% higher expression than group housed males [$P < 0.0001$]. Additionally, non-parenting males showed 31% higher levels of aromatase expression that those in group-housed conditions [$P = 0.0001$].

3.3. Brain isotocin receptor gene expression

Two-way ANOVA showed a significant effect of parenting



(caption on next page)

[$eta^2 = 0.12$, $F_{1,37} = 5.37$, $P = 0.02$] and sex [$eta^2 = 0.11$, $F_{1,37} = 5.57$, $P = 0.02$] on relative IT receptor gene expression, and no interaction between sex and parenting [$eta^2 = 0.01$, $F_{1,37} = 0.58$, $P = 0.45$]. Overall, males displayed 17% higher IT receptor gene expression than females (Fig. 2B), and individuals currently caring for eggs displayed 19% higher levels than those not actively caring for eggs.

Results from the one-way ANOVA comparing males across treatments, including the group-housed condition, showed a significant

Fig. 2. Aromatase, IT and AVT V1a gene expression (GE) in males and females as a function of parental status. Relative abundance of aromatase (A), IT receptor (B), and AVT V1a receptor (C) gene expression levels in the context of parenting ('Active') vs. non-parenting ('Non-Active'). Females are represented in red and males are represented in blue. Box plots show the interquartile range (IQR) of each group analyzed with whiskers extending to 1.5× the IQR. Horizontal lines represent medians, and diamonds represent means. Scattered points within each boxplot represent the individual values used to generate each plot. Relative gene expression is shown by dividing each individual value by the mean of the Non-Active males within the gene. Relative gene expression was calculated via the equation: Fold change = $2^{-\Delta\Delta Ct}$, by comparison to the reference gene beta actin. Lowercase letters denote significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

main effect of treatment [$eta^2 = 0.45$, $F_{2,26} = 10.63$, $P < 0.001$]. Post hoc analyses indicated parenting males show a 22% increase in isotocin receptor expression compared to non-parenting males and a 74% increase compared to group housed males [$P = 0.04$, $P < 0.001$]. Additionally, non-parenting males show 42% higher relative levels of IT receptor gene expression that those in group-housed condition [$P = 0.01$].

3.4. Brain arginine vasotocin receptor (V1a) gene expression

Results from the two-way ANOVA showed no effect of parenting [$eta^2 = 0.01$, $F = {}_{1,37}$ 0.52, $P = 0.47$] or sex [$eta^2 = 0.01$, $F = {}_{1,37}$ 0.52, $P = 0.78$] on relative v1a gene expression (Fig. 2C). Additionally, no interaction between sex and parenting was present [$eta^2 = 0.01$, $F_{1,37} = 0.06$, $P = 0.8$]. Comparing males across treatments, results from one-way ANOVA showed a significant effect of the group-housing condition [$eta^2 = 0.54$, $F_{1,26} = 15.86$, $P < 0.0001$]. Males kept in group-housed conditions show 10% less relative V1a gene expression than males in the active spawning group [$P < 0.001$], and also 8% less expression than males in the non-active group [$P < 0.001$].

3.5. Circulating steroid hormone levels

Due to the non-normal distribution (not meeting Shapiro-Wilks criterion despite transformations) of circulating E2 levels in females, a Kruskal-Wallis test was used in evaluating the effect of breeding status within females. Reproductive status (active parenting vs. non-parenting) had a significant effect on estradiol levels [df = 1, $chi^2 = 5.64$, $P = 0.02$], where females that were actively parenting displayed 2-fold higher levels of circulating estradiol levels than non-active females.

Levels of estradiol within males met normality criteria and there were no significant effects of parenting status on estradiol levels [$eta^2 = 0.30$, $F_{2,15} = 3.29$, $P = 0.07$]. Due to limitations in blood sampling of group-housed males, only actively spawning and non-spawning males were compared.

In evaluating the difference between the sexes in circulating levels of estradiol, a Kruskal-Wallis test was used. Overall, females displayed 29-fold higher levels of circulating estradiol than males, consistent with previously reported results [$chi^2 = 12.80$, $P < 0.001$] (Deangelis and Rhodes, 2016).

In females, which displayed higher aromatase gene expression and also higher circulating estradiol, aromatase gene expression and circulating estradiol were positively correlated [$r_{14} = 0.75$, $P < 0.001$]. This correlation was not observed in males [$r_{12} = 0.13$, $P = 0.65$].

Circulating levels of 11KT in females did not meet normality criteria. Hence, a Kruskal-Wallis test was used to determine the effect of reproductive status on 11KT levels. There was no effect of reproductive status on 11KT levels within females [$chi^2 = 2.04$, $P = 0.15$]. Distributions of 11KT in males met normality criteria and so a one-way ANOVA was used to evaluate the treatment effect. Similarly, within males, there was no effect of parenting status on circulating levels of 11KT [$eta^2 = 0.15$, $F_{1,13} = 2.3$, $P = 0.15$]. Males displayed 9.3-fold higher 11KT than females [$chi^2 = 20.3$, $P < 0.001$], supporting previously published data (Deangelis and Rhodes, 2016). Additionally, males displayed a positive correlation between circulating 11KT levels and estradiol levels [$r_{12} = 0.72$, $P < 0.001$], a correlation that did not occur in females [$r_{14} = -0.40$, $P = 0.11$]. There were no significant relationships between any of the circulating steroid hormone concentrations and behaviors measured in this present study.

4. Discussion

Here, we present the first study to our knowledge, showing that brain aromatase and IT receptor gene expression levels differ in actively parenting vs. non-parenting males. Both males and females displayed increased aromatase and IT receptor gene expression while actively caring for eggs compared to non-active pairs, suggesting that both brain aromatase and IT signaling are important in the regulation of parental care in the anemonefish *A. ocellaris*. Our results are consistent with a growing literature suggesting that IT/OT signaling plays a key role in paternal care across multiple species of vertebrates. Several studies have shown that fatherhood often requires an active shift in neurological features in order to support the paternal behavioral repertoire (De Jong et al., 2009; Kent and Bell, 2018; Kim et al., 2014; Wu et al., 2014). We conclude that aromatase and isotocin receptor gene expression vary as a plastic response to the dynamic behavioral shift required for parental care.

4.1. Brain aromatase gene expression

Brain aromatase gene expression was higher in both males and females that were actively parenting compared to those that were not parenting (Fig. 2A). This suggests that synthesis of E2 from T within the brain may be an important factor in the regulation of fathering behavior in males. E2 treatments are known to engender parental behaviors in both male and female rodents (Rosenblatt and Ceus, 1998; Wynne-Edwards, 2001). In the California mouse, conversion of T to E2 within the brain by aromatase is critical for high levels of male parental care, where gonadectomized males treated exogenously with T or E2 displayed greater paternal care than controls, and simultaneous use of an aromatase inhibitor blocked the positive effect of T treatment while having no effect on the rise in care in response to E2 (Trainor and Marler, 2002). These findings implicate brain aromatase activity in paternal care. Our results support this idea, and further establish that the effect is local within the brain, as circulating E2 levels were unrelated to parental status and uncorrelated with brain aromatase levels in the male anemonefish.

Simultaneously higher levels of circulating E2 complicate the explanation for higher aromatase levels in females. In addition to displaying a small number of parental behaviors, they were also likely in a physiological state of producing the next batch of eggs to be laid. Consistent with this idea, circulating E2 levels were much higher in actively parenting females compared to non-parenting females. Moreover, within females, circulating estradiol levels and brain aromatase gene expression were highly correlated. Nuclear estrogen receptors, which bind to the promoter region on the aromatase gene, positively regulate aromatase gene expression (Callard et al., 2001). Therefore, if circulating E2 reflected E2 concentrations in the brain, then it is possible that the E2 increased brain aromatase gene expression through this positive feedback mechanism.

Previously, we have shown that circulating E2 levels vary during the reproductive cycle in female *A. ocellaris*, and that E2 levels are higher on day 6 of the egg brooding period than on the day the eggs were laid or when no eggs were present (Deangelis and Rhodes, 2016). Taken together, these data are consistent with the idea that the higher E2 in parenting females is related to the physiological state of egg production rather than the small number of parental behavioral acts they perform.

Nevertheless, whether high brain aromatase activity in females is related to egg development and/or parental care is unclear, and future work exploring each possibility in a specific context is needed to fully understand the role of E2 during female parental care in *A. ocellaris*.

Overall our data suggest that within males, local conversion of T to E2 within the brain by aromatase may work in the promotion of parental care. Unlike many other models in which fathering behavior co-occurs with territorial defense, aggression and nest building, in *A. ocellaris* these other behaviors are largely absent during parental care in an aquarium setting, strengthening the conclusion that local E2 production is key to fathering behavior specifically. Based on the literature, we hypothesize brain aromatase activity is a precursor to the upregulation of the IT signaling system which promotes paternal care, although the causal relationship remains unclear and will require further work to establish. In females, we propose increased circulating E2 and correlated brain aromatase levels work to promote reproduction and/or parental care.

4.2. Isotocin receptor gene expression

Results here indicate that IT receptor abundance increased in response to parental care. This is consistent with a growing body of literature highlighting the importance of not only the function of OT/IT neurons, but also the expression of OT/IT receptors throughout the brain. In humans, paternal variation is associated with genetic variation at the OT receptor gene (Feldman, 2012), while in female prairie voles natural variation in OT receptor expression during development in the nucleus accumbens positively correlates with alloparental behavior (Keebaugh and Young, 2011). Moreover, OT receptor knockout mice perform fewer maternal behavioral acts than controls (Keebaugh et al., 2015). In addition, a study in the bi-parental convict cichlid showed that blockade of IT signaling inhibited the rise in paternal effort over the egg incubation period, and that single fathers displayed increased activation of IT neurons within the parvocellular layer of the POA (O'Connell et al., 2012). Additionally, we have previously shown that blockade of the IT receptor dramatically reduces male parental effort in the anemonefish *A. ocellaris* (Deangelis et al., 2017). The consistency of this pattern across species supports recent work showing that the OT/IT signaling system is highly conserved across evolutionary history in both form, and in the functional way it facilitates parental care (Dulac et al., 2014; Goodson, 2008; O'Connell and Hofmann, 2012; Royle et al., 2012).

During reproduction the female brain undergoes a dramatic reorganization of the OT/IT signaling system (Gimpl and Fahrenholz, 2001). Oestrus and pregnancy provide a physiological mechanism for OT production in females, where in rodents, the hormones of pregnancy are critical for the production of OT receptors in the amygdala and hypothalamus and the onset of parenting (Broad et al., 1999). Results presented here suggest that the male brain also undergoes a shift of the IT signaling system. Parenting males display higher levels of paternal care, and correspondingly higher levels of IT receptor expression, which we speculate may be a response of increase E2 within the brain as mediated by higher aromatase activity. While we did not explore the causal relationship between circulating E2, brain aromatase activity, and IT signaling, future studies investigating these relationships may yield a more specific understanding of the interplay between IT and brain E2 levels as a function of aromatase activity during paternal care.

4.3. AVT receptor gene expression

While the AVT signaling system in the brain has generally been thought of as promoting behaviors in males related to reproductive success, few studies have been able to isolate a role in behaviors directed at offspring care. The lack of a direct relationship between AVT signaling and specific parental behaviors may be due in part to the confounding influences of co-occurring behaviors such as territorial defense and aggression. For example, in the three-spined stickleback fish, AVT peptide levels in the brain were highest in the most aggressive males that were also actively caring for eggs (Kleszczyńska et al., 2012). Our results suggest that this difference may be related to the heightened aggression rather than paternal care. In anemonefish males, which display low aggression but high levels of parental care, no differences in AVT receptor gene expression were observed (Fig. 2C). Moreover, our previous report found that blockade of AVT using an AVT V1a receptor antagonist actually increased male parental care, perhaps suggesting an opposite role for AVT signaling in directly suppressing paternal care rather than increasing it (Deangelis et al., 2017). However, in that report, we provided an alternative hypothesis that AVT regulates paternal care indirectly by drawing attention away from offspring care and toward aggression and/or territorial defense. Consistent with this idea, blockade of AVT reduced aggression in a different context (Deangelis et al., 2017; Yaeger et al., 2014). Moreover there is a large body of literature on the role of AVT in aggression across species (Foran and Bass, 1999; Kleszczyńska et al., 2012; Kline et al., 2011; Semsar et al., 2001). This interpretation is consistent with our data, given that in our study no nest defense or vigilance behaviors were displayed, hence no differences in AVT V1a expression should have been observed. Future work is needed to directly test the hypothesis that AVT signaling specifically facilitates vigilance during parental care.

4.4. 11KT

While high androgens have been traditionally viewed as inhibitory to high levels of parental care, this ‘challenge hypothesis’ has less support outside of the avian studies where it originated (Wingfield et al., 1990; Wynne-Edwards and Timonin, 2007). In these avian studies, males spawn seasonally, and are required to build nests, defend territories and compete for mates prior to reproduction. Hence, high androgens are critical in regulating future reproductive success during this combative pre-reproductive period. However, in other studies, androgens are necessary for parental care. In the California mouse, high T levels are necessary for paternal care (Trainor and Marler, 2001), and male sunfish in better body condition had higher circulating androgens during parental care (Magee et al., 2006). Finally, some species display levels of parental care that are independent of circulating androgen levels, such as the smallmouth bass (Hanson et al., 2009), three spined stickleback (Páll et al., 2002), and our previous work in *A. ocellaris,* in which circulating 11KT were unrelated to breeding status in males (Deangelis and Rhodes, 2016). The data presented here are consistent with previous results, suggesting that parental care in *A. ocellaris* is likely independent of circulating levels of the main bioactive androgen, 11KT. On the other hand, it is possible that brain levels are regulated locally rather than from the blood as suggested by a study in blue-banded gobies where intracerebroventricular injections of carboxolene, which blocks the conversion of testosterone to 11KT within the brain, reduced male parental care, highlighting the importance of androgen hormone synthesis within the brain in regulation parental care (Pradhan et al., 2014). Taken together, although some minimal level of 11KT may be necessary for fathering, it is unlikely that varying levels of circulating 11KT contribute to the onset of fathering, but future work is needed to investigate the possible contributions of local synthesis of 11KT in the brain.

4.5. Limitations

One important limitation of this study is that we measured gene expression in the whole brain and thus do not know how the patterns of gene expression might vary in different brain regions. The lack of differences in AVT V1a receptor gene expression between groups, for example, could be because gene expression increased in certain regions and decreased in others, thus obscuring differences when collapsed. In many of the previous studies showing effects of AVT receptors on social

behaviors, AVT receptors were quantified in a brain region specific manner (Kline et al., 2011). On the other hand, the genes we measured, aromatase, IT receptors and AVT receptors are known to be widely distributed throughout the brain (Forlano et al., 2001; Greenwood et al., 2008; Hausmann et al., 1995; O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012). Hence, the whole brain differences we observed herein provide the rationale for future studies to identify and characterize regional specificity.

Similarly, the brain is composed of many different types of cells (e.g., neurons, glia, endothelial cells) and we analyzed gene expression from the entire mixture, so we do not know whether the gene expression patterns we found were influenced primarily by variation in one or multiple cell types. For example, evidence suggests aromatase is synthesized in radial glial cells (Forlano et al., 2001; Tong et al., 2009), hence it is likely that the gene expression differences we observed for aromatase reflect changes in radial glial cells or astrocytes. Similarly, the nonapeptide receptors are likely expressed in neurons, but may also be expressed on astrocytes and microglia. Future work is needed to explore the gene expression patterns in a cell type specific fashion to confirm the cellular origin of the signals reported herein.

5. Conclusion

This is the first report to which we are aware that compared gene expression levels of aromatase, IT receptors, and AVT V1a receptors in the whole brains of actively parenting vs. non-parenting male and female *A. ocellaris*. Additionally, we measured the circulating hormone concentrations of E2 and 11KT. Our results indicate that aromatase and IT receptor gene expression are dynamically regulated by parental status, higher during parenting than not parenting in both males and females. Furthermore, while hormone levels varied by sex, and E2 was higher in actively parenting females, parental care in *A. ocellaris* appears to be largely independent of circulating sex steroid concentrations. Taken together, our results highlight the importance of IT signaling and brain aromatase activity in the regulation of parental care in the anemonefish, *A. ocellaris*.

Acknowledgements

We would like to thank Michael Saul for his expertise in primer design and Carolyn Butts Wilmsmeyer for her statistical acumen. This work was supported by the Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under project number ILLU 875-952, and by start-up funds, indirect costs recovered from federal grants, and gifts to J.S.R.

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