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Active feminization of the preoptic area occurs independently of the gonads in *Amphiprion ocellaris*

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ABSTRACT

Sex differences in the anatomy and physiology of the vertebrate preoptic area (POA) arise during development, and influence sex-specific reproductive functions later in life. Relative to masculinization, mechanisms for feminization of the POA are not well understood. The purpose of this study was to induce sex change from male to female in the anemonefish *Amphiprion ocellaris*, and track the timing of changes in POA cytoarchitecture, composition of the gonads and circulating sex steroid levels. Reproductive males were paired together and then sampled after 3 weeks, 6 months, 1 year and 3 years. Results show that as males change sex into females, number of medium cells in the anterior POA (parvocellular region) approximately double to female levels over the course of several months to 1 year. Feminization of gonads, and plasma sex steroids occur independently, on a variable timescale, up to years after POA sex change has completed. Findings suggest the process of POA feminization is orchestrated by factors originating from within the brain as opposed to being cued from the gonads, consistent with the dominant hypothesis in mammals. Anemonefish provide an opportunity to explore active mechanisms responsible for female brain development in an individual with male gonads and circulating sex steroid levels.

1. Introduction

In most sexually reproducing animals, the brain is masculinized or feminized in a manner consistent with the gonads (Panzica and Melcangi, 2016). Across vertebrates, the most robust and reliable brain sexual dimorphisms are found in the preoptic area (POA) of the hypothalamus, consistent with the central role of the POA in regulating reproductive physiology and behavior (Bleier et al., 1982; Boyd et al., 1992; Breedlove, 1992; Grober et al., 1991; Moore et al., 2000; Viglietti-Panzica et al., 1986; Wade and Crews, 1991). For example, female rats have approximately twice the number of neurons co-expressing kisspeptin and dopamine in the anteroventral periventricular nucleus of the POA, a region which is known to control gonadotropin releasing hormone (GnRH) cell function (Clarkson and Herbison, 2011; Gu and Simerly, 1997; Popolow et al., 1981; Simerly et al., 1985; Wiegand and Terasawa, 1982). In contrast, male rats have approximately 3 times the number of neurons in the sexually dimorphic nucleus (SDN) of the POA as females (Gorski et al., 1980), a difference hypothesized to function in regulating male sexual behaviors (De Jonge et al., 1989).

It is well established in rats that masculinization of the POA is initiated from a spike in gonadal androgens released from the testes during a critical window of development (Feder and Whalen, 1965; Grady et al., 1965; Phoenix et al., 1959; Whalen and Edwards, 1967). Because the ovary does not secrete a significant amount of estrogen during the neonatal period to influence brain development, for a long time it was thought that feminization of the POA has no active mechanisms but rather occurs by default in absence of androgens (Greco and Payne, 1994; Lamprecht et al., 1976). However, a number of studies have challenged the notion that feminization of the brain is a passive process. For example, several studies have found that removal of the ovary at various stages of pre and postnatal development in rodents and other vertebrates including fishes defeminize certain aspects of neurochemistry, neuroanatomy and behavior (Bimonte et al., 2000; Fitch et al., 1991; Mack et al., 1993; Melo and Ramsdell, 2001; Painson et al., 1992; Yates and Juraska, 2008). Moreover, the current consensus in mammals is that the female brain undergoes substantial reorganization during puberty under the control of ovarian hormones (Juraska and Willing, 2017). Hence, certain aspects of brain feminization, especially during postnatal development, appear to require

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ovarian-derived hormones. In addition, recent evidence in rodents suggests that the chemical and cellular processes which contribute to active feminization of the POA during embryonic development can originate from within the brain itself independent of the ovary. For example, in absence of gonadal steroids during embryonic development, feminization of the POA requires active suppression of gene expression within brain cells (Nugent et al., 2015).

Unraveling the mechanisms of active feminization of the POA is difficult in rodents, because there is no known trigger such as a spike in gonad-released steroid levels which initiates the process (Bakker and Baum, 2008; Bakker et al., 2003; McCarthy and Arnold, 2011). However, there are many vertebrates which share homologous brain regions and neurochemistry as mammals, but which do not have sex chromosomes and where sex is determined by environmental factors (Francis, 1992). Among these organisms, anemonefish are particularly promising for discovering mechanisms involved in active feminization of the brain because anemonefish first mature as males, and then change sex into females as adults (Fricke and Fricke, 1977). This provides some unique advantages. In anemonefish, feminization of the brain is inarguably active. An adult male brain transforms into an adult female brain. Moreover, in anemonefish, the signals which initiate feminization of the brain are known and easy to manipulate. *Amphiprion ocellaris*, the false percula clownfish or common anemonefish live in small groups with one alpha female, one beta male, and zero to a few lower ranking non-reproductive males (Mitchell, 2005). A male transforms into a female if the female is removed from the group (Casas et al., 2016; Fricke and Fricke, 1977; Godwin, 1994; Godwin and Thomas, 1993; Madhu and Madhu, 2006) or if two males are paired together (Fricke, 1983). Finally, feminization is easier to study in anemonefish than in mammals since it occurs in the adult animal rather than in the embryo which is more difficult to manipulate.

This is the first study to induce sex change in the laboratory in anemonefish as a model for exploring active feminization of the brain. The goal was to determine if the preoptic area of the hypothalamus (POA) is sexually dimorphic in *A. ocellaris* as it is in many other vertebrate species including *A. melanopus* (Elofsson et al., 1997), and then to establish the time course for changes in POA neuroanatomy, gonadal histology and circulating sex steroid levels during the course of sex change from male to female in the anemonefish, *Amphiprion ocellaris*. Results provide the foundation for using the novel anemonefish model to discover mechanisms of active brain feminization.

2. Materials and methods

2.1. Animals

All fish used in this study were offspring bred in-house from brood stock obtained from ORA (Oceans Reefs and Aquariums, Fort Pierce, FL). Fish were kept in pairs, consisting of one dominant female and one subordinate male in 20-gallon tall (24″ × 12″ × 16″) or 25-gallon cube (18″ × 18″ × 18″) aquariums. Only fish that had been established this way for over 3 years, and had been observed spawning at least once per month were used in this study.

A total of 42 reproductive males and 4 adult reproductive females were used. Body lengths and weights of the females ranged from 5.4 to 7.5 cm and 2.9 g–10.3 g. The males were divided into two groups. The first group (n = 34) were paired together randomly to initiate sex change (see below). At the time when they were paired, these males ranged 4.4–6.1 cm and 1.6–4.0 g. The second group was sampled directly from their established reproductive pair without any social manipulations. These males (n = 8) ranged 5.4–6.9 cm and 2.7–5.5 g.

2.2. Husbandry

Aquariums were integrated via plumbing to a large circulating filtration system. Each tank contained one 6″ terra-cotta pot, to serve as the nesting site. 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 0700 h and off at 1900), and specific gravity of 1.026. Fish were fed at least twice a day with a mix of New Life Spectrum Marine Formula (Homestead, Florida) and Golden Pearls (from Brine Shrimp Direct, Ogden, Utah), with frozen adult brine shrimp (San Francisco Bay Brand Newark, CA), and mysis shrimp (Hikari Sales, USA) supplemented approximately once per week. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

2.3. Experimental design

Actively reproducing male *A. ocellaris* (n = 34) were removed from their mates and randomly paired together in an aquarium without any other fish. A total of 17 pairs were formed this way and remained undisturbed until they were sampled at 4 different time-points: 3 wk (n = 7 pairs), 6 months (n = 4 pairs), 1 year (n = 4 pairs), and 3 years (n = 2 pairs). As soon as the fish were paired together they were observed carefully for an hour, and checked every day thereafter until dominance status had clearly been established with one dominant and one subordinate fish. This determination was based on subjective assessment by an experienced care-taker (J.S.R.) and involved direct observations of agonistic and posturing interactions between the fish or lack thereof, and location of the fish with respect to the nesting site (terra cotta pot) (Iwata et al., 2008; Yaeger et al., 2014).

2.3.1. Blood hormone assays

Between 1400 and 1600 h on the day of sampling, blood was taken from the lateral caudal vein following published methods (DeAngelis and Rhodes, 2016). 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. In some of the smaller individuals, it was not possible to obtain sufficient blood for analysis. The blood was used to measure plasma estradiol (E2) and 11-ketotestosterone (11KT), because levels are highly sexually dimorphic as detailed in DeAngelis and Rhodes (2016).

2.3.2. Body and brain

Immediately following the blood draws, the fish were euthanized by cervical transection and the brains were carefully dissected from the skull. The body and brain were placed in separate vials containing 4% paraformaldehyde chilled at 2 °C and then stored in the refrigerator. The following morning, the tissues were moved to 30% sucrose solution for preservation and returned to the refrigerator until sectioning.

2.4. Gonadal histology

Bodies were removed from the 30% sucrose after 48 h, and placed in Tissue Mounting Media (Tissue-Tek O.C.T. Compound, Sakura, Finetek) and frozen in the −80 freezer. Bodies were then serially sectioned at 40 µm in the sagittal plane using a cryostat (Thermo Scientific, Microm HM 550) and placed directly on subbed slides (i.e., slides treated with gelatin so sections would stick). Slides were stored in the −80 freezer until staining. The day before staining, slides were left out overnight to defrost then stained as follows: they were washed briefly in distilled water, and then placed in Harris Hematoxylin solution (Sigma, HHS-32) for 6 min, followed by 5 min of washing in tap water by putting the slide tray in a beaker under the faucet for a U shaped flow stream. Slides were then washed in 1% HCL in 70% ETOH for 30 s. Sections were again washed in running tap water for 1 min, then washed in 1.54% lithium carbonate in dH2O for 1 min, followed by another wash with running tap water for 5 min. Then sections were rinsed in 95% alcohol 5 times (1–3 dips in five separate wells of alcohol) and then counterstained in an eosin solution for 30 s to 1 min. The eosin solution contained 1 g Eosin Y, 0.1 g Phloxine B, 110 ml dH2O, 780 ml 95% ETOH,

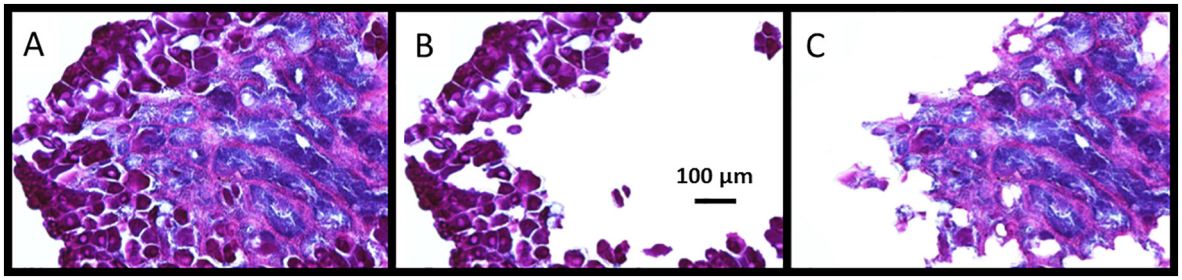

Fig. 1. Illustration of the method for measuring percentage of testicular versus ovarian tissue in the gonad. A. The original image containing both non-vitellogenic oocytes and testicular tissue before parcellation. B. An example of how non-vitellogenic oocytes are parcellated from the gonad section using Adobe Photoshop. C. Testicular tissue parcellated from the gonad section.

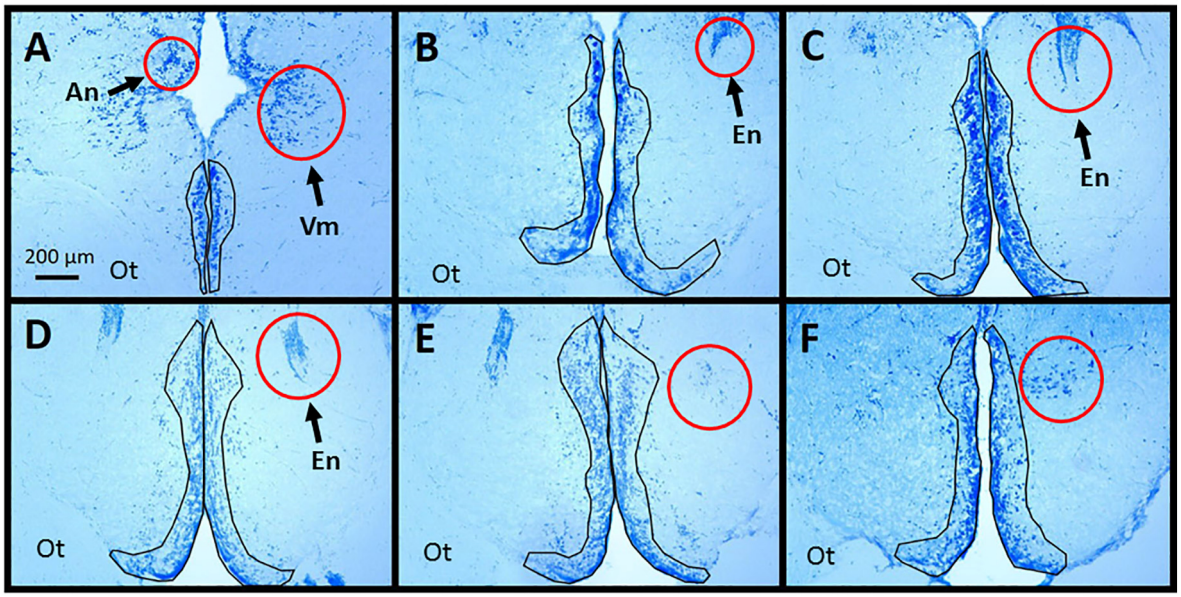

Fig. 2. Illustration of the landmarks that were used to identify the different regions of the POA. The schematic diagram at the top indicates the approximate locations along the caudal to rostral axis of the divisions between the posterior (p), middle (m), and anterior (a) POA. The thickness of the entire posterior section ranges from approximately 400–520 µm, the middle 320–360 µm and the anterior 240–280 µm. A. The POA is outlined in black and landmarks are indicated by a red circle. Moving from caudal to rostral, the beginning of the posterior POA is marked by the prominent appearance of the ventromedial thalamic nucleus (Vm) and the end of the anterior thalamic nucleus (An). The POA at this point does not extend to the lower boundaries of the brain as it does in the other sections. B. The end of the posterior POA is marked by the first appearance of the entopeduncular nucleus (En). The bottom of the POA extends to the ventral surface of the brain and begins to fan out. C. The beginning of the middle POA is indicated by a slightly larger En. D. The end of the middle POA is indicated by the last section with a prominent En. At this point the En is also disconnected from the top portion around the ventricle. E. The beginning of the aPOA is marked by the last remnants of En cells. It is also marked by lateral expansion of the POA dorsally. F. The end of the aPOA is marked by the peak appearance of an unknown population of cells in place of En. Ot indicates the optic tract. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4 ml glacial acetic acid. To dehydrate, slides were placed in 95% ETOH for 6 min, then 100% ETOH for 6 min, followed by xylenes for 10 min. Slides were cover-slipped with Permount Mounting Medium.

Sections containing the gonads were photographed using an Axiocam (MRc 5) camera mounted to a Zeiss light microscope running Axio Imager software. For each individual, 20 sections through the gonads were chosen at random for analysis from the series. Photographs were taken at 100× or 50× total magnification, depending on the size of the gonads. The photos were analyzed using Adobe Photoshop CS6 software. First the gonad was outlined, and then the Quick Selection tool was used to differentiate testicular tissue from ovarian tissue semi-automatically with manual correction until the tissue types were differentiated sufficiently (Fig. 1). Ovarian tissue was further divided into containing non-vitellogenic oocytes or vitellogenic oocytes. The area fraction of the gonad covered by each of these tissue types (% testicular tissue, % ovarian-non-vitellogenic, % ovarian-vitellogenic) was evaluated for each of the sampled sections. In addition, the three sections which contained the largest area of gonads for each individual were photographed at 25× magnification to provide a relative measure of gonad size.

2.5. Brain histology

Brains were removed from the 30% sucrose after 24 h, and placed carefully in Tissue Mounting Media (Tissue-Tek O.C.T. Compound, Sakura, Finetek) so that the brain was oriented vertically along the rostro-caudal axis and frozen in a −80 freezer until sectioning. Brains were then serially sectioned at 14 µm in the coronal plane using the cryostat and placed directly on subbed slides. Slides were stored in the −80 freezer until staining. The day before staining, sections were left out overnight to defrost. Slides were stained as follows: 2 min in 70% ethanol (ETOH), 2 min in 50% ETOH, 5 min in 0.2 M phosphate buffer solution (PBS), and 5 min in 1% Periodic Acid in deionized water (dH2O). Next, the slides were placed in the Methylene Blue Azure II stain for 7 min. The stain was a mix of 0.16 g methylene blue, 0.1 g azure II, 1.7 g sodium phosphate dibasic anhydrous, and 0.56 g of potassium phosphate monobasic per 100 ml of dH2O. Slides were then dehydrated as follows: 50% ETOH for 30 s, 70% ETOH for 1 min, 95%

ETOH for 1 min, 6 dips in 100% ETOH, then xylenes for 10 min followed by cover slipping with Permount Mounting Medium.

2.6. Stereology

Alternate sections of the entire preoptic area (POA) were examined using a Zeiss Axio Imager light microscope with motorized stage and focus running Stereo Investigator software. Sections were examined at 200× magnification to determine the 2-dimensional area and total number of cells. The POA was divided into three separate regions, the anterior, middle, and posterior regions following published cichlid brain atlases (Burmeister et al., 2009; Fernald and Shelton, 1985) and the atlas of the gilthead seabream brain as references (Muñoz-Cueto, 2001). Specific landmarks were used to objectively delineate the ends of the different regions of the POA (Fig. 2). The anterior portion contains mostly the parvocellular region of the POA. The middle and posterior regions contain mostly magnocellular and gigantocellular regions, respectively, though there is a fair degree of overlap due to the regions not being perfectly separated on the rostro-caudal axis (Burmeister et al., 2009; Fernald and Shelton, 1985; Muñoz-Cueto, 2001; O'connell and Hofmann, 2011; O'Connell and Hofmann, 2012). As there are multiple different cell types within the regions which vary by cell size, each cell that was counted was categorized as small (diameter < 6 μm), medium (diameter between 6 and 12 μm), or large (diameter > 12 μm). Examples of cells that fell into each of these categories is shown in Fig. 3. Counting was performed using the Fractionator Probe in Stereo Investigator. The grid perimeters entered were 50 um for the x-axis and 50 um for the y-axis.

2.7. Plasma sex steroid hormone 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) (DeAngelis and Rhodes, 2016). Each plasma sample was diluted 1:30 in assay buffer as done in DeAngelis and Rhodes (2016), 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) following manufactures' instructions.

2.8. Statistical methods

Data were analyzed using SAS (version 9.4) Proc MIXED and SAS Proc GLM. Proc MIXED, was used in cases where there were unequal sample sizes between groups since it uses restricted maximum likelihood for parameter estimation rather than sums of squares (Littell et al., 2006; Pinheiro and Bates, 2006). $P < 0.05$ was considered statistically significant. Data were considered normally distributed if residual distribution showed skewness between −1 and 1 and kurtosis between −2 and 2. Least Significant Difference (LSD) tests, i.e., simple *t*-tests using the pooled mean square error from the overall analysis, were used for post-hoc tests of pair-wise differences between means. Proc GLM was used to generate partial η^2 values for ANOVA effect sizes. For paired *t*-tests, effect sizes are given as Cohen's d, calculated as the mean difference divided by the standard deviation of the difference scores.

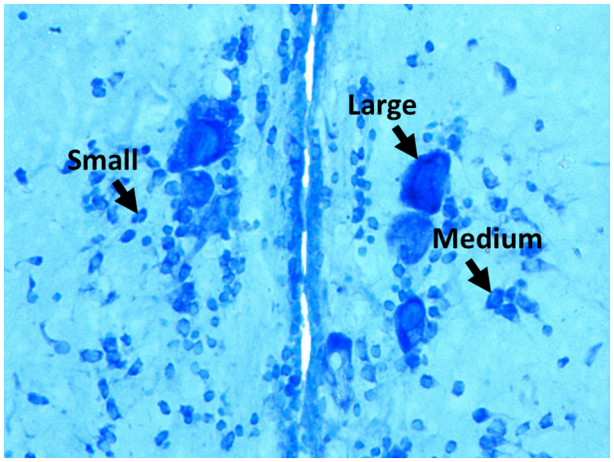

Fig. 3. Illustration of the three sizes of cells in the POA that were counted separately using the optical fractionator stereological method. This section was taken from the posterior region of the POA.

2.8.1. Dominance and body size

A paired *t*-test was used to evaluate whether the subordinate fish was significantly smaller than the dominant fish at the start of the sex change experiment. Growth (i.e., change in length and weight between start of experiment and sampling time) was analyzed by 2-way ANOVA considering time-point (3-wk, 6-mon, 1-yr, 3-yr) as one factor and dominance status (dominant or subordinate) as another factor.

2.8.2. Gonadal histology

Percent testicular tissue was analyzed by 1-way ANOVA comparing intact males to dominant and subordinate individuals sampled at 3-wk, 6-mon, 1-yr, and 3-yr (9 levels). Percent testicular tissue was also correlated with relative gonad size with and without including body length as a covariate using Pearson's correlation and linear regression respectively.

2.8.3. Neuroanatomy

Pearson's correlations were used to evaluate relations between volume of the POA sub-regions, body size (length and weight of the fish), and numbers of small, medium and large cells in the POA sub-regions. Number of small, medium, and large cells in each POA sub-region, as well as volume of each region were analyzed separately using 1-way ANOVA comparing intact males to females and to dominant and subordinate individuals sampled at the 4 time-points (10 levels). Analyses were also conducted including volume of the POA sub-region and body length as a covariate.

2.8.4. Circulating sex steroid hormones

Concentration of 11KT and E2 in the plasma was analyzed by 1-way ANOVA comparing intact males to females and to dominant and subordinate individuals sampled at the 4 time-points (10 levels).

3. Results

3.1. Dominance is determined by body size

Within the first few minutes when the two males were placed together, they approached each other and in some cases fought with each other by biting and ramming. Dominance was clearly established after the first few days in all but 1 of the 17 pairs. In this one pair, the fish were by chance the same length and weight when they were paired together and continued to fight and posture up until the time when they were sampled at 3 weeks. Because dominance could not be established, they were excluded from further analysis. Among the remaining 16 pairs, the behaviorally dominant fish was larger than the subordinate in 15 out of the 16 (Fig. 4A,B). In the one pair where the subordinate fish was larger, the difference was small, 1 mm and 0.8 g. The mean difference in length and weight between the dominant and subordinate fish was significant by paired *t*-test (for length, Cohen's $d = 1.19$, $t_{15} = 4.8$, $P = 0.0003$; weight, Cohen's $d = 1.04$, $t_{15} = 4.2$, $P = 0.0008$). These results confirm, as previously reported for this species that dominance is largely determined by body size (Iwata et al., 2008).

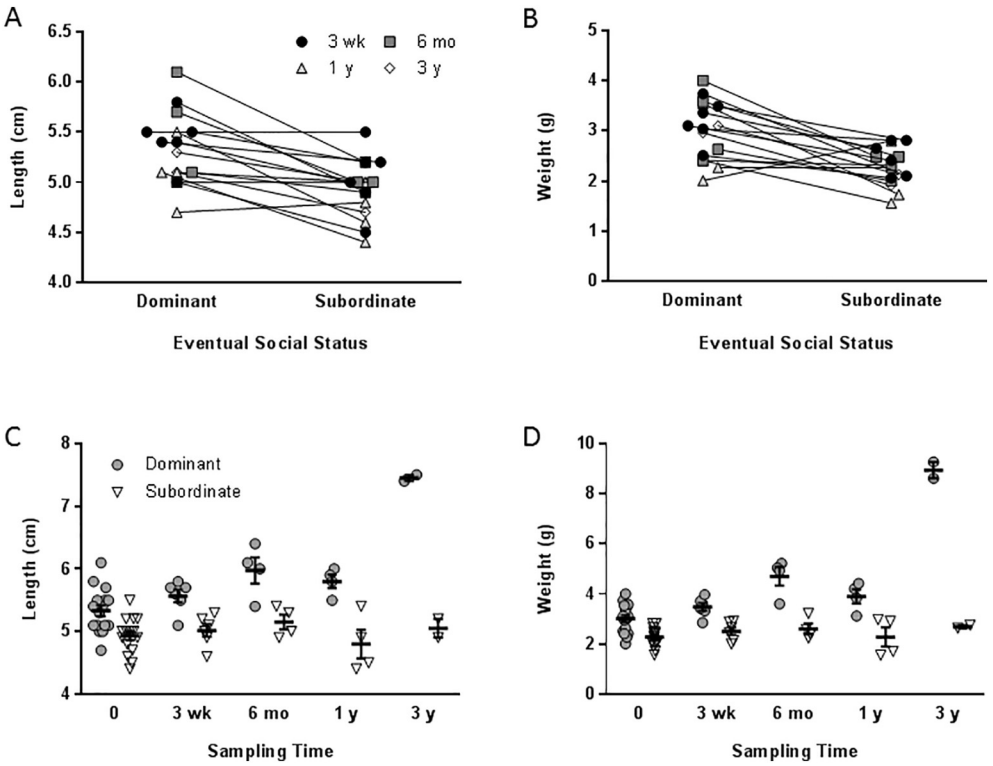

Fig. 4. Initial body size determines dominance status and subsequent growth rate. A and B. Length and weight of the males at the time when they were originally paired together at the start of the sex change experiment. Fish that became dominant are shown on the left and subordinate on the right with lines connecting the two individuals in each pair. Data points are coded with separate symbols to represent their future sampling time points. C and D. Body length and weight plotted against sampling time. Means are shown as horizontal bars within the scatter plot with standard errors bars shown in the vertical position. Time point zero is the same data as shown in panels A and B. Dominant fish grew substantially in body size over the 3 years, whereas growth in subordinate fish was not detectable.

3.2. Dominant individuals grow significantly faster than subordinates

Over the course of the sex change experiment, the dominant member of the pair grew whereas the subordinate member did not (Fig. 4C,D). This was indicated by a significant effect of time-point ($\eta^2 = 0.82$, $F_{3,24} = 37.1$, $P < 0.0001$; $\eta^2 = 0.87$, $F_{3,24} = 55.2$ $P < 0.0001$), dominance status ($\eta^2 = 0.74$, $F_{1,24} = 67.6$, $P < 0.0001$; $\eta^2 = 0.82$, $F_{1,24} = 111.1$, $P < 0.0001$) and the interaction of time-point and dominance ($\eta^2 = 0.75$, $F_{3,24} = 24.5$, $P < 0.0001$; $\eta^2 = 0.87$, $F_{3,24} = 55.4$, $P < 0.0001$) status on growth of the fish in length and weight (i.e., change in length and weight between start of experiment and sampling time), respectively. Posthoc tests comparing growth differences between dominant and subordinate fish were significant at the 6-mon ($P = 0.01$, $P < 0.0001$), 1-yr ($P = 0.0002$, $P = 0.0003$) and 3-yr ($P < 0.0001$, $P < 0.0001$) time-points but not 3-wk ($P = 0.15$, $P = 0.28$) for length and weight, respectively. Within dominant fish, growth was different between each pair of time-points (all $P < 0.007$) except between 6 months and 1 year ($P > 0.16$ for length and weight). Within subordinate fish, growth was not different between any of the time-points, indicating little growth had occurred (all $P > 0.15$). These results confirm as previously reported for this species and other related species of anemonefish that the alpha member of the group grows significantly faster than the subordinate individuals as it differentiates into the female sex (Fricke, 1983; Iwata et al., 2008).

3.3. Feminization of the gonad occurs on a variable time-scale after a protracted period of gonadal regression

3.3.1. Sex differences

Consistent with previous reports for anemonefish (Fricke and Fricke, 1977; Godwin, 1994; Rattanayuvakorn et al., 2006), reproductive males displayed an ambisexual gonad containing an average of 71% (± 0.026 SEM) testicular tissue and 29% non-vitellogenic oocytes. The non-vitellogenic oocytes were found in the periphery of the gonad and were wrapped around the testicular tissue. None of the males had vitellogenic oocytes. Reproductive females displayed 100% vitellogenic-oocytes and no detectable testicular tissue (Fig. 5F,I,J).

3.3.2. Gonadal sex change

Only 3 episodes of a gonadal sex change occurred out of the 17 pairs of males that were formed. Here, gonadal sex change is defined by the presence of at least some vitellogenic oocytes in the gonad of an individual which was once male. Based on these criteria, the larger, behaviorally dominant member of one of the pairs sampled at 6 months changed gonadal sex as indicated by 100% vitellogenic oocytes in the gonads. One of the pairs from the 1-year time-point was observed spawning and caring for a batch of viable eggs, indicating a sex change had occurred. Histological analysis showed that the larger, behaviorally dominant individual changed gonadal sex into a female and the smaller subordinate member regained status as a male. The female's gonads contained 100% vitellogenic oocytes. In one of the pairs from the 3-year time point, the larger, dominant fish had several vitellogenic oocytes accounting for 13% of the gonad (Fig. 5H). The remaining gonad was comprised of non-vitellogenic oocytes (87%), i.e., no testicular tissue could be detected. This result demonstrates that gonadal sex change in *A. ocellaris* occurs on a variable time-scale and requires some unknown stimulus to occur which is individual or context specific.

Results from the one-way ANOVA on percent testicular tissue across all the different groups of males and ambisexual individuals (individuals in any of the sex change groups which had not changed gonadal sex) yielded a significant effect of group ($\eta^2 = 0.84$, $F_{8,28} = 22.1$, $P < 0.0001$). Post-hoc analysis indicated that within each time-point no differences in gonadal composition were found between subordinates and dominant individuals (all $P > 0.05$; Fig. 5J). Hence subordinates and dominants were collapsed within time-points. The resulting 1-way ANOVA on the collapsed data also showed a significant effect of group ($\eta^2 = 0.79$, $F_{4,32} = 30.1$ $P < 0.0001$). All pair-wise differences between time-points and reproductive males were significant ($P < 0.05$) except 1-yr vs. 3-yr ($P = 0.93$). The 3 subordinate individuals which were paired with a fish that changed gonadal sex (as described above) were placed in separate groups (with n = 1 each) so they could be compared to reproductive males and other members of

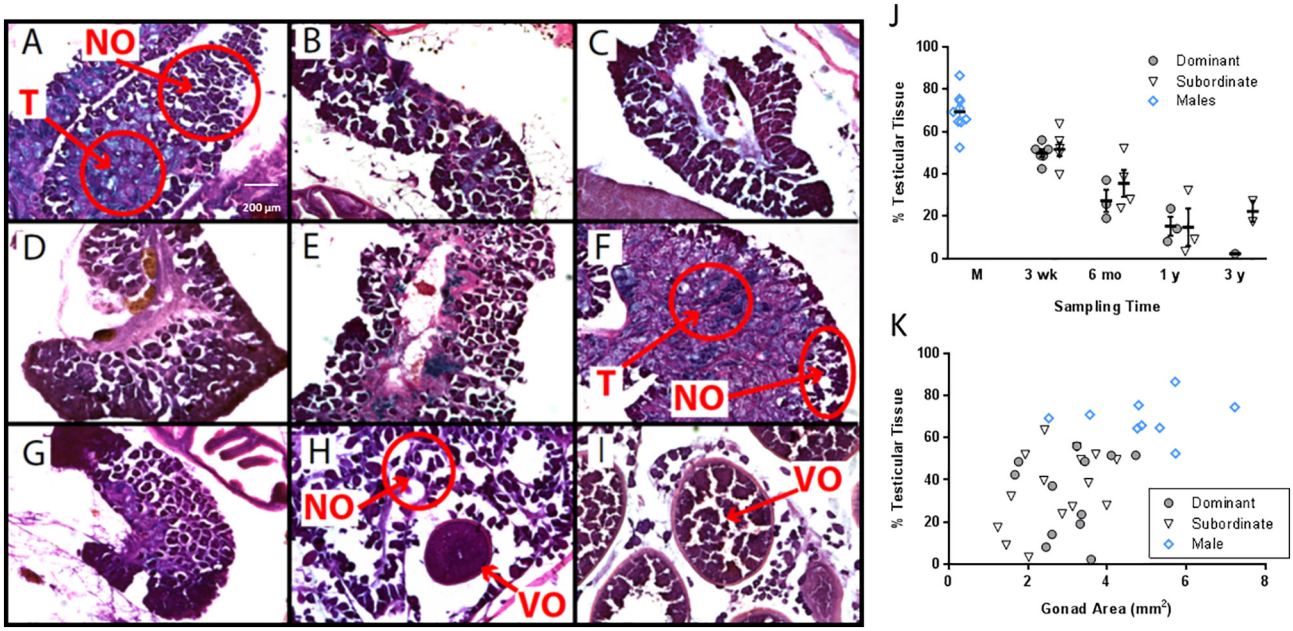

Fig. 5. Gonadal sex change is preceded by a long period of stasis during which time testicular tissue regresses. A–I. Representative microscopic images taken at 50× magnification of sagittal sections through the gonads stained with Hematoxylin and Eosin. A–C. Fish sampled at 3 wks, 6 mon and 1 year, respectively. Over time, testicular tissue (T) is reduced leaving mostly non-vitellogenic oocytes (NO) remaining. This process occurs in both subordinate and dominant members of the pair. D. A fully regressed gonad from the subordinate member of a pair sampled at 3 years whose partner displayed a similarly regressed gonad. E. A gonad from the subordinate member of a pair sampled at 3 years whose partner displayed a few vitellogenic oocytes (VO). The gonad appears in the initial stages of redeveloping testicular tissue. F. A gonad from a fully mature reproductive male showing the ambisexual gonad with a majority of testicular tissue in the center enveloped by a layer of non-vitellogenic oocytes on the periphery. G. A fully regressed gonad from the dominant member of a pair sampled at 3 years with zero vitellogenic oocytes, and very little testicular tissue present. H. A gonad from the dominant member of a pair sampled at 3 years displaying only a few vitellogenic oocytes. I. A gonad from a fully mature reproductive female showing large numbers of vitellogenic oocytes. J. Percentage of testicular tissue in the gonad plotted against sampling time, with males (M) shown to the left. Means are shown as horizontal bars within the scatter plot with standard errors bars shown in the vertical position. Percentage of testicular tissue decreases over time, with no differences between subordinate and dominant members of the pair within each time-point until 3 years when subordinates show evidence of regaining testicular tissue while testicular tissue in dominant members continues to shrink. K. Percentage of testicular tissue in the gonad plotted against gonad size. The variables are correlated suggesting that reduced percentage of testicular tissue in the gonad is a result of lost testicular tissue rather than the addition of non-vitellogenic oocytes. Females which display large gonads with zero testicular tissue are excluded. Each group is shown as a separate symbol or color.

their time-point. The fish that were not yet spawning (at 6-mon and 3-yr time-points) showed percent testicular tissue similar to other fish in their time-point ($P > 0.05$) and reduced testicular tissue as compared to reproductive males (both $P < 0.004$). In contrast, the fish that was spawning (1-yr) showed the opposite pattern, increased testicular tissue as compared to the 1-yr time-point ($P = 0.0007$) and not different from males ($P = 0.09$). These results demonstrate that testicular tissue is reduced in both the subordinate and dominant fish before gonadal sex change occurs. They further demonstrate that testicular tissue re-develops in subordinate fish only after the dominant fish has transformed gonadal sex completely and is ready to spawn.

Without correcting for body length, the relative size of the gonad was positively correlated with the percent testicular tissue ($r = 0.59$, $P < 0.0001$). After correcting for body length (partial $r = 0.44$, $F_{1,35} = 13.5$, $P = 0.0008$), the partial correlation remained similar to the uncorrected estimate (partial $r = 0.57$, $F_{1,35} = 22.2$, $P < 0.0001$; Fig. 5K). Taken together these results indicate that after two males are paired together, the gonads of both the dominant and subordinate fish slowly regress in unison to a point of mostly non-vitellogenic oocytes with some small fraction of testicular tissue present. The progression occurs slowly over the course of a year or longer. At some point between 3 weeks and 6 months, the fish are capable of changing gonadal sex, except it occurs with low frequency. In only 1 of 4 pairs did it occur after 6 months, and in only 1 out of 4 pairs after 1 year. Even after 3 years, the 2 pairs that were sampled had not completed the process, as indicated by zero or very few vitellogenic oocytes (Fig. 5G,H). Hence, fish can remain in a state of gonadal regression as defined by a small gonad comprised of mostly immature oocytes for years before gonadal sex change occurs (Fig. 5C,D,E,G,J).

3.4. Sexual dimorphism in the anterior POA (aPOA): Females display greater numbers of medium size cells and reduced volume relative to males

3.4.1. Sex differences in cell number

Without adjusting for volume of the aPOA, females displayed 1.8 times as many medium cells in anterior region as males ($\eta^2 = 0.42$, $F_{1,8} = 5.8$, $P = 0.04$; Fig. 6A,C,D,F,G). All Pearson's correlations between numbers of cells and volume were above 0.64 (see Table 1). Correlations were significant for small and medium cells in the anterior region, medium cells in the middle region, and small cells in the posterior region. After correcting for volume, the effect size was larger ($\eta^2 = 0.68$, $F_{1,7} = 14.6$, $P = 0.007$) with females displaying 2.1 fold more medium cells than males. This indicates significantly greater total number and density of medium cells in the aPOA in females as compared to males. No other statistically significant sex differences in cell numbers were detected.

3.4.2. Sex differences in volume of the POA sub-regions

Without adjusting for body length, males and females displayed similar volumes of the anterior, middle, or posterior regions of the POA. However, significant correlations were observed between body length and volume of the POA sub-regions in females (Fig. 7A). Pearson's r values were 0.93 ($P = 0.02$), 0.91 ($P = 0.03$), and 0.99 ($P = 0.001$) for the anterior, middle, and posterior regions, respectively. Slightly

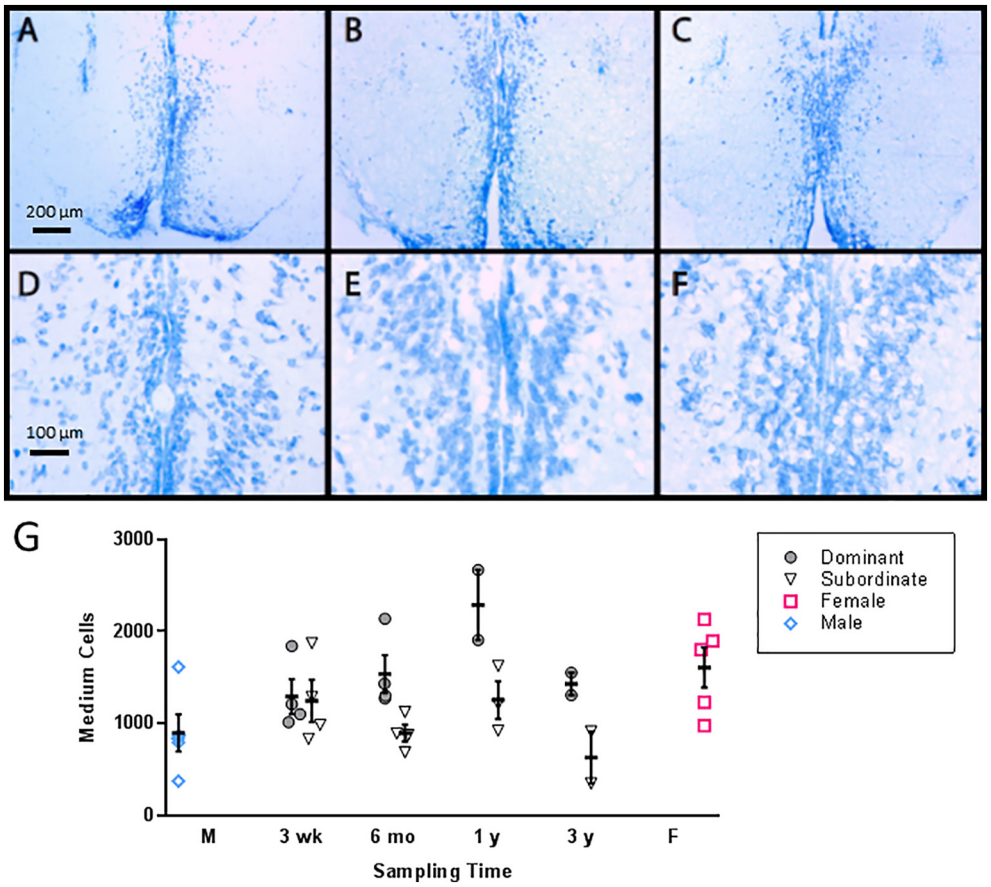

Fig. 6. Active feminization of the anterior POA (aPOA). A–C. Representative sections through the anterior POA from a reproductive male, the dominant member of a pair sampled at 6 months, and a reproductive female, respectively. Photos were taken at 50× magnification. D–E shows the same region zoomed in at 100× magnification. Females have a significantly greater number of medium size cells (diameter between 6 and 12 µm) than males in this region. The dominant fish at 6 months has an intermediate number of medium size cells. G. Total number of medium cells in the aPOA plotted against time-point with reproductive males (M) on the left and reproductive females (F) on the right. Means are shown as horizontal bars within the scatter plot with standard errors bars in the vertical position. Significant differences were observed between dominant and subordinate at the 6-mon and 1-yr time-points. None of the subordinate groups differed from males, whereas dominant members from the 6-mon and 1-yr time-points displayed significantly greater numbers of medium cells than males. Females were significantly different from 6-mon subordinate and 3-yr subordinate groups.

Table 1
Correlations between number of cells and volume of the POA among intact females.

Cell type	Region of the POA					
	Anterior		Middle		Posterior	
	r	P-value	r	P-value	r	P-value
Small	0.98	0.003	0.829219	0.083	0.879462	0.049
Medium	0.92	0.029	0.907282	0.033	0.641462	0.243
Large	0.84	0.074	0.763406	0.133	0.789679	0.112

weaker correlations were observed for body weight, 0.91 (P = 0.03), 0.86 (P = 0.06), and 0.98 (P = 0.003). After adjusting for body length by analysis of covariance, males displayed approximately 50% larger volume of the anterior portion of the POA as compared to females ($\eta^2 = 0.70$, $F_{1,7} = 16.0$, P = 0.005; Fig. 7B). No significant sex differences were observed for the middle or posterior regions after accounting for body length. Taken together, these results establish sexual dimorphism in the aPOA of *A. ocellaris*, with females showing greater numbers of medium size cells and reduced volume relative to body length.

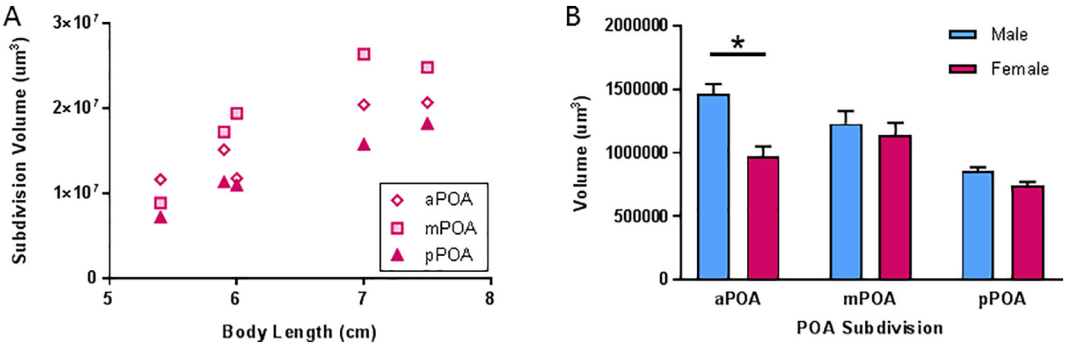

Fig. 7. Males display larger volume of the aPOA per unit body length. A. Volume of the POA sub-regions (anterior, middle and posterior) plotted against body length in the 5 reproductive females (4 original fish plus the 1 that changed sex and was spawning at the 1-year time-point). The two variables are significantly correlated in each region. B. Least-square mean ± SE POA volume (adjusted for body length via analysis of covariance) shown separately for males and females for each of the three POA sub-regions. Males have a larger aPOA than females per unit body length. Without correcting for body length, there is no difference. * indicates statistically significant (P < 0.05).

Table 2
Least-square adjusted mean (± SE) number of small, medium and large cells in each of the three sub-regions of the POA shown separately by group. Means are adjusted for volume of the sub-regions by analysis of covariance.

			aPOA		mPOA		pPOA	
Small cells	Subordinate	3 wk	21,488.00	± 1535.68	19,658.96	± 1623.22	11,888.48	± 1247.64
		6 mo	19,523.22	± 1555.18	16,362.52	± 1673.10	10,534.50	± 1283.86
		1 y	19,929.42	± 1787.34	16,999.56	± 1885.96	10,453.04	± 1507.90
		3 y	24,076.00	± 2211.02	19,534.62	± 2337.80	13,334.54	± 1771.48
	Dominant	3 wk	19,933.14	± 1570.34	17,706.94	± 1647.28	14,185.50	± 1250.28
		6 mo	17,212.02	± 1846.18	14,673.02	± 1644.90	9030.18	± 1240.16
		1 y	16,712.06	± 2185.02	15,639.58	± 1875.84	8058.86	± 1446.46
		3 y	19,233.92	± 2152.76	16,746.72	± 2386.42	15,986.62	± 2137.96
	Male		23,582.00	± 1366.80	19,706.24	± 1458.84	13,090.00	± 1114.10
	Female		23,522.00	± 1404.32	19,425.62	± 1609.18	14,539.72	± 1166.84
Medium cells	Subordinate	3 wk	1303.14	± 188.60	1366.50	± 203.54	1069	± 156.47
		6 mo	986.86	± 190.99	1260.18	± 209.80	890.46	± 161.01
		1 y	1157.84	± 219.50	1173.60	± 236.48	932.72	± 189.11
		3 y	775.72	± 271.54	1163.48	± 293.14	905.36	± 222.16
	Dominant	3 wk	1402.62	± 192.85	1982.54	± 206.56	1159.08	± 156.80
		6 mo	1234.06	± 226.72	1415.20	± 206.26	929.98	± 155.53
		1 y	2177.34	± 268.34	1334.54	± 235.22	1160.92	± 181.40
		3 y	1448.60	± 264.38	1066.36	± 299.24	1315.9	± 268.12
	Male		860.54	± 167.86	1100.18	± 182.92	1076.88	± 139.72
	Female		1706.36	± 172.46	1179.72	± 201.78	1117.12	± 146.34
Large cells	Subordinate	3 wk	39.92	± 22.32	246.58	± 61.93	53.07	± 17.69
		6 mo	19.82	± 22.60	211.52	± 63.96	48.24	± 18.21
		1 y	0	± 25.98	117.11	± 71.96	39.36	± 21.38
		3 y	16.77	± 32.14	171.03	± 89.34	60.30	± 25.12
	Dominant	3 wk	69.32	± 22.82	297.5	± 76.11	85.15	± 17.73
		6 mo	13.45	± 26.83	155.47	± 63.12	63.41	± 17.59
		1 y	22.95	± 31.76	152.49	± 71.57	50.43	± 20.51
		3 y	126.48	± 31.29	120.87	± 92.00	61.65	± 30.32
	Male		9.60	± 19.87	162.25	± 55.64	57.23	± 15.80
	Female		38.94	± 20.41	259.74	± 62.88	87.00	± 16.55

3.5. Feminization of the aPOA occurs on a predictable time-scale following the initiation of sex change and is completed by approximately 1 year

3.5.1. Number of medium cells in the aPOA

Results from the one-way ANOVA on number of medium cells yielded a significant effect of group ($\eta^2 = 0.55$; $F_{9,25} = 3.4$, $P = 0.007$; Fig. 6G). Posthoc analyses comparing dominant to subordinate at each time-point showed no differences at 3-wk ($P = 0.87$), but significant difference at 6-mon ($P = 0.03$) and 1-yr ($P = 0.01$) and trend at 3-yr despite the small sample of $n = 2$ per group ($P = 0.06$). Males were significantly different from 6-mon dominant ($P = 0.03$) and 1-yr dominant groups ($P = 0.0004$). Females were significantly different from 6-mon subordinate ($P = 0.01$) and 3-yr subordinate ($P = 0.008$) groups. The same analyses above were conducted after correcting for volume of the POA by entering it as a covariate. The covariate was significant ($\eta^2 = 0.29$; $F_{1,24} = 5.5$, $P = 0.028$) as was group ($\eta^2 = 0.57$; $F_{9,24} = 3.5$, $P = 0.007$; Table 2). Posthoc analysis comparing dominant to subordinate at each time-point showed no differences except at 1-yr ($P = 0.006$). The 1-yr dominant group was also the only group that significantly differed from reproductive males ($P = 0.0003$; see Table 2). Taken together, results establish that the number of medium cells in the aPOA increase to female levels over a period of 6-mon to 1-year in *A. ocellaris*.

3.5.2. Volume of the aPOA

Results showed a significant effect of the length covariate ($\eta^2 = 0.07$; $F_{1,24} = 5.0$, $P = 0.035$), and a significant effect of group ($\eta^2 = 0.52$; $F_{9,24} = 2.9$, $P = 0.017$). However, the pattern was not consistent with a feminization process. No significant differences were detected between subordinate and dominant individuals within time-points. The only evidence for feminization was that reproductive females and 3-yr dominant individuals displayed the smallest volumes corrected for body length, and both were significantly lower than the 1-yr subordinate group (both $P < 0.03$; see Table 3). Taken together, results suggest the reduction in volume of the aPOA in females versus males occurs after gonadal sex change has occurred and is unrelated to changes in dominance status, growth, or numbers of medium cells in the aPOA.

3.6. Feminization of plasma sex steroid levels occurs on a variable time-scale after a protracted period when sex steroid levels remain within the male range

3.6.1. 11KT

A main effect of group was detected in the 1-way ANOVA ($\eta^2 = 0.73$; $F_{8,18} = 6.17$, $P = 0.0007$; Fig. 8A). As expected, levels of 11KT were significantly higher in males than females ($P = 0.0002$). Levels of 11KT were significantly lower in both subordinate and dominant individuals at the 3-week time-point as compared to males (both $P < 0.01$), but were not different from each other. At the 6-month time-point 11KT concentration in subordinates returned to male levels and was significantly higher than dominant individuals ($P = 0.02$), which remained low. At 1 year, a similar trend was observed. Although the difference between subordinates and dominants was not significant ($P = 0.06$), subordinates displayed similar levels of 11KT as reproductive males ($P = 0.81$) whereas dominant individuals displayed lower 11KT than males ($P = 0.01$). At 3 years, only one dominant individual was measured which showed a high 11KT level similar to males. Taken together, these data show that before gonadal sex change is initiated, plasma concentration of 11KT fluctuate between male-typical and low male levels, but rarely reach female-typical low levels until vitellogenic oocytes appear in the gonads.

3.6.2. E2

A main effect of group was detected in the 1-way ANOVA ($\eta^2 = 0.75$; $F_{8,19} = 7.2$, $P = 0.0002$). Females displayed much higher E2 than all other groups with no overlap with other individuals (all $P < 0.002$). No other pair-wise differences between groups were

Table 3
Least-square adjusted mean (± SE) volume of the three sub-regions of the POA shown separately for each group. Means were adjusted for length of the fish by analysis of covariance.

		aPOA		mPOA		pPOA	
Subordinate	3 wk	18,497,538	± 1,638,362	18,350,756	± 1,871,272	12,669,526	± 1,320,288
	6 mo	17,347,000	± 1,518,078	15,028,898	± 1,733,136	10,532,588	± 1,222,824
	1 y	22,190,190	± 2,082,108	19,557,578	± 2,377,780	12,346,290	± 1,677,656
	3 y	16,716,044	± 2,105,888	15,363,414	± 2,402,562	11,477,456	± 1,695,142
Dominant	3 wk	15,766,098	± 1,399,486	13,200,830	± 1,594,946	9,499,916	± 1,125,324
	6 mo	21,692,486	± 1,485,058	14,379,202	± 1,689,410	8,966,186	± 1,191,974
	1 y	19,060,370	± 1,988,960	13,113,016	± 1,857,410	10,939,860	± 1,310,506
	3 y	11,827,708	± 3,141,832	7,797,656	± 3,573,228	8,978,998	± 2,521,112
Male		18,721,476	± 1,268,468	14,862,674	± 1,446,844	10,481,374	± 1,020,830
Female		13,663,596	± 1,604,910	14,684,330	± 1,823,418	8,982,356	± 1,286,524

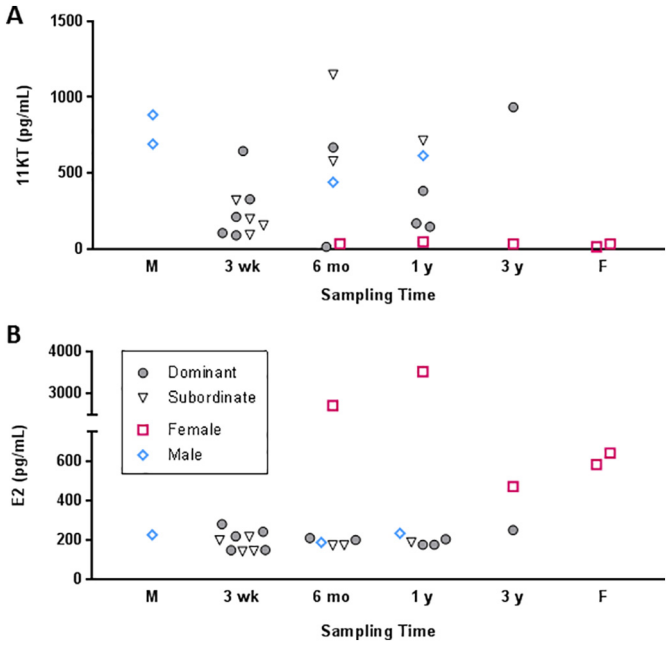

Fig. 8. Plasma sex steroid levels. A. Individual plasma levels of 11-KT in picograms per ml are plotted against time-point with reproductive males on the left and reproductive females on the right. Means are shown as horizontal bars within the scatter plot with standard errors bars in the vertical position. Individuals which changed sex from male to female, as indicated by the presence of vitellogenic oocytes in the gonads, are represented with the female symbol and their partner as male in the graph. C. Same graph for E2. Note that several samples are missing because of failed blood collection. Some segregation between dominant and subordinate fish in 11-KT levels occurs at 6 mon and 1 yr, but E2 levels never segregate until vitellogenic oocytes appear in the gonad.

detected. These data suggest that circulating E2 levels remain low, at male-typical levels until vitellogenic oocytes finally appear in the gonads, after which E2 increases to female-typical levels (DeAngelis and Rhodes, 2016).

4. Discussion

Active feminization of the POA during sex change from male to female was induced and recorded for the first time in the anemonefish, *Amphiprion ocellaris*. Results reveal that feminization of the POA occurs first, sometimes years before feminization of the gonad is initiated, and proceeds independently of circulating sex steroid hormone levels. After males are paired together, and dominance is established, the number of medium size cells in the aPOA increases in the dominant fish to female levels over the course of several months to a year (Fig. 6). Meanwhile the number of medium cells in the POA of the subordinate fish remains the same, and similar to male levels. At the same time when the brain anatomy is diverging between the subordinate and dominant fish, the gonads degenerate in unison, both becoming smaller with reduced testicular tissue and consisting of similar amounts of non-vitellogenic oocytes (Fig. 5). Since the fish are not reproducing, energy to maintain and produce viable gonads may be diverted elsewhere. The degenerated gonad with reduced testicular tissue and associated male levels of circulating 11KT and E2 can last for years before vitellogenic oocytes appear, E2 rises and 11KT falls to female levels (Fig. 8). Taken together, these results suggest that feminization of the brain is orchestrated by processes from within the brain itself, and that only after the brain transformation is complete, do fish have the capability to feminize their gonads. However, feminization of the gonad does not happen automatically. Instead, it appears that the fish can remain with a female brain and behavior but with ambisexual, regressed male-like gonads and circulating sex steroid levels for years, waiting for some unknown signal before completing the process and producing vitellogenic oocytes. Only after feminization of the gonads is initiated, and vitellogenic oocytes begin to appear, do the fish display high circulating E2 levels and plummeted levels of 11-KT typical of females.

4.1. Feminization of the aPOA occurs independently of the gonads

The observation that feminization of the brain precedes the gonad is consistent with early studies in rodents and song birds which concluded that a female brain and behavior develops in absence of gonadal influences (Arnold, 1975, 2017; Feder and Whalen, 1965; Grady et al., 1965; Phoenix et al., 1959; Whalen and Edwards, 1967), but inconsistent with the Japanese quail where adult female behavior is dependent on estradiol signaling during embryonic development (Adkins, 1979; Balthazart et al., 1992; Jost, 1983; Panzica et al., 1998; Panzica et al., 1987). The apparent lack of input from the gonads in feminization of the POA in anemonefish is in stark contrast to what happens during masculinization of the POA in mammals which is completely dependent on the formation of testicular tissue, and relies on gonadal steroid signals for orchestrating the processes (Davis et al., 1996; Jacobson et al., 1981; McCarthy, 2017). It is also very different from the chain of events known to underlie sex change from female to male in fishes, in which gonadal sex hormones fluctuate in concert with the morphological transformations and are sufficient to induce the body color, body size and brain sexual dimorphisms (Bhandari et al., 2003; Cardwell and Liley, 1991; Godwin, 2010; Grober et al., 1991; Kroon and Liley, 2000; Nakamura et al., 1989). Our results are consistent with current thinking in mammals that active feminization of the POA during embryonic development originates from within the brain itself (McCarthy and Arnold, 2011). The brain mechanisms could use circulating hormones as substrates, but their activity is controlled by processes within the brain, such as aromatase locally released and controlled by surrounding neurons and brain circuits.

4.2. Comparative neuroanatomy

The number of medium size cells in the aPOA approximately doubled as a male transformed into a female. This region contains the parvocellular nucleus in fishes which is considered homologous to the sexually dimorphic regions of the POA in mammals, birds, reptiles, and amphibians (Bleier et al., 1982; Boyd et al., 1992; Breedlove, 1992; Grober et al., 1991; Moore et al., 2000; O'connell and Hofmann, 2011; O'Connell and Hofmann, 2012; Saha et al., 2015; Simerly et al., 1985; Viglietti-Panzica et al., 1986; Wade and Crews, 1991). One of the most reliable sex differences in the POA across species of mammals is the anteroventral periventricular nucleus (AVPv) (Davis et al., 1996; McCarthy and Arnold, 2011) which shows 2 to 3 times the number of dopamine neurons in female versus male rats (Simerly et al., 1985). A subset of these dopamine neurons in the AVPv are known to co-express kisspeptin and estrogen receptor alpha (Esr1) and play a crucial role in ovulation via direct regulation of GnRH neuron activity (Clarkson and Herbison, 2011; Smith et al., 2006). Female mice have approximately 10-fold more of these kisspeptin/dopamine neurons in the AVPv than males (Clarkson and Herbison, 2006). Catfish also display a similar sexual dimorphism in number of dopamine neurons in the aPOA (Saha et al., 2015), and similar to mammals, these dopamine neurons influence GnRH activity (Bryant et al., 2016). There is some debate currently about whether these aPOA dopamine neurons contain kisspeptin in teleost fishes and whether kisspeptin has any role in regulating GnRH neuron function (Kim et al., 2014; Nakajo et al., 2017; Parhar et al., 2004). Taken together, the current literature would suggest that the increased number of medium cells in the aPOA (Fig. 6) might be partially composed of dopamine neurons or other neurons which express Esr1 and which control GnRH neuron function.

In addition to dopamine neurons or Esr1 neurons which regulate GnRH neurons, the increased number of medium cells in the aPOA could include GnRH neurons themselves, which are between 6 and 12 μm in diameter in cichlids and in *A. melanopus* (Davis and Fernald, 1990; Elofsson et al., 1997), and are found in this location. On the other hand, a previous study found fewer GnRH cells in females than males in a related anemonefish species, *A. melanopus* (Elofsson et al., 1997). Other possibilities include arginine vasotocin and isotocin neurons, which are the teleost homologs of arginine vasopressin and oxytocin and are found in these locations. These neuropeptides are crucially involved in regulating aggression, territorial defense and parenting all of which are sexually dimorphic in *A. ocellaris* (DeAngelis et al., 2017; DeAngelis et al., 2018). However, in goldfish the cells which contain these neuropeptides are between 24 and 30 μm in diameter, larger than the medium cell category used herein (Reaves Jr and Hayward, 1980). Antibodies to all these neuropeptides are available and can be used in future studies to uncover the identity of the medium cells that become more numerous in female aPOA.

Despite displaying greater numbers of medium cells in the aPOA than males (Fig. 6), females actually displayed a smaller aPOA volume than males after correcting for variation due to body length (Fig. 7B). This difference, however, did not emerge until after gonadal sex change was initiated because subordinate and dominant members of the pairs displayed similar body-length corrected aPOA volumes at each of the time-points (Table 3). Perhaps the dimorphism in aPOA volume requires high circulating estradiol levels which only happen after vitellogenic oocytes are present in the gonad (Fig. 8B). The difference in volume could arise from decreased number, size and/or dendritic arborization of a sub-population of cells in females which we were not able to parse out here due to the methodology which does not distinguish cell types. The functional significance is unclear at this point. The difference could be homologous to the SDN in rats which is larger in males than females and functions in regulating male sexual behavior (De Jonge et al., 1989; Gorski et al., 1980). Future work is needed to identify the origin of the sexual dimorphism in aPOA volume, the functional significance (if any), and the extent to which development is dependent on sex steroids.

4.3. Anemonefish life history

Our timeline for the process of gonadal sex change in *A. ocellaris* is perfectly aligned with the only other report of sex change in the laboratory for this species (Madhu and Madhu, 2006). Madhu and Madhu (2006) found it took approximately 6 months to a year before the male displayed vitellogenic eggs after the female was removed, and 1 to 1 1/2 years before it spawned, consistent with our estimates. It is possible that this relatively long timeline is specific for *A. ocellaris* since estimates for *A. melanopus* in field experiments were 3 weeks to a month (Godwin, 1994), and the original report by Fricke and Fricke in 1977 found that after removal of the dominant female in *A. akallopisos* it took < 63 days for the beta-male to change into a female (Fricke and Fricke, 1977). On the other hand, the histological evidence provided by both these papers showed the female with only non-vitellogenic oocytes, no vitellogenic oocytes were present, consistent with our data, and the idea that gonadal sex change had not yet occurred. Therefore, it is possible that the 1 to 1 ½ year time line is common to anemonefish. It is notable that our estimate is more in line with some of the original field observations which found that it took > 1 year for the male to change into the female after the dominant female disappeared naturally from a group of *A. clarkii* (Moyer and Nakazono, 1978).

The explanation for why a fish would not complete the gonadal sex change automatically after the brain feminization process has occurred is not known and is an interesting mystery. The long time-lag may reflect a trade-off between devoting energy toward somatic growth versus egg production. *A. ocellaris* and their sister species *A. percula* can live up to 30 years, and grow to approximately 11 cm (Buston and García, 2007; Moyer and Nakazono, 1978). Larger fish produce more eggs, and therefore devoting energy toward growth until reaching a size large enough to produce a larger number of eggs instead of reproducing right away could pay off in the long run. The decision to increase in size rather than lay eggs right away might also be beneficial for solidifying the alpha position in the dominance hierarchy and might protect the fish from prematurely changing sex. Anemonefish are not thought to be able to naturally change back to male after reproducing as a female (Fricke and Fricke, 1977) (though ovaries can be masculinized with an aromatase antagonist, Nakamura et al., 2015), and two female *A. ocellaris* are not compatible on a single territory (Mitchell, 2005). Therefore, if the gonad feminizes prematurely and the dominant female returns, the outcome would be disastrous for the fish, as the smaller female would be booted out of the territory or killed by the larger female. Hence, there are several reasons, related to the peculiar life history of anemonefish, why delaying gonadal sex change even for years might still be adaptive in terms of reproductive fitness.

An alternative possibility from the fitness explanation is that the large variation in timing of gonadal sex change in *A. ocellaris* is a by-product of the laboratory environment and unnatural. This explanation would posit that the fish would have changed gonadal sex in a predictable way if in the field. Evidence against this idea comes from the field sex change experiments in *A. bicinctus* and *A. melanopus* in which the gonadal histology results are consistent with the laboratory timeline (Casas et al., 2016; Godwin, 1994) and the timeline reported for natural sex change in *A. clarkii* from field observations (Moyer and Nakazono, 1978). A big difference between our laboratory setting and the field is that fish are exposed to recirculating water and potentially pheromones or hormones in the water from other breeding females in the system could suppress the other females from finishing their gonadal development. In nature, groups of *A. ocellaris* are typically far enough away from each other that they are unlikely to be able to detect each other's presence (Mitchell, 2005). On the other hand, in our experience and personal communication with individuals that breed *A. ocellaris* on a commercial basis, having a pair spawning in a system, so long as they cannot see each other, tends to stimulate other pairs in the

system that have not spawned yet to start spawning. That would suggest that the laboratory environment should speed up not slow down gonadal sex change and decrease the time to first spawning. Thus, we conclude that the long delays and relatively large variation in the timeline for gonadal sex change in the species is a natural phenomenon related to the unique life history of anemonefish, strict genetic monogamy, isolated small populations, long lifespan, and independence of brain and gonadal sex.

5. Conclusions

Our results demonstrate that the anemonefish brain is capable of actively transforming and reorganizing itself from the male type to the female type long before vitellogenic oocytes appear in the gonads. Fluctuating plasma levels of gonadal hormones do not appear necessary for feminization of the brain, as E2 remained low and consistent as the brain feminized, and 11KT shifted down in both dominant and subordinate fish in association with reduced testicular tissue. It is still possible the gonads provide substrates required for brain feminization, such as testosterone, which is then aromatized in the brain for initiating the neuroanatomical changes. Alternatively, testosterone could be supplied by cells from within the brain (Mensah-Nyagan et al., 1996). Future studies where males are gonadectomized before they are paired together would be able to arbitrate between these possibilities. The conclusion that brain feminization in anemonefish is orchestrated from signals originating from within the brain rather than the gonads is consistent with the dominant hypothesis in mammals that the POA feminizes in absence of gonadal influences (McCarthy and Arnold, 2011). Anemonefish provide a window for discovering the cellular, biochemical and physiological mechanisms underlying active feminization of the brain in vertebrates.

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