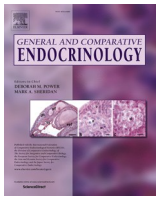
General and Comparative Endocrinology 333 (2023) 114185

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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



New cells added to the preoptic area during sex change in the common clownfish *Amphiprion ocellaris*

Coltan G. Parker [a], Sarah E. Craig [b], Abigail R. Histed [b], Joanne S. Lee [b], Emma Ibanez [b], Veronica Pronitcheva [b], Justin S. Rhodes [a,b,c,*]

[a] *Neuroscience Program, University of Illinois, Urbana-Champaign, 405 N Mathews Ave, Urbana, IL 61801, USA*
[b] *Beckman Institute for Advanced Science and Technology, University of Illinois, Urbana-Champaign, 405 N Mathews Ave, Urbana, IL 61801, USA*
[c] *Department of Psychology, University of Illinois, Urbana-Champaign, 603 E Daniel St, Urbana, IL 61801, USA*

ARTICLE INFO

Keywords:
Anemonefish
BrdU
Preoptic area
Sexual differentiation
Neurogenesis
Sequential hermaphroditism
Sex change

ABSTRACT

Sex differences in cell number in the preoptic area of the hypothalamus (POA) are documented across all major vertebrate lineages and contribute to differential regulation of the hypothalamic-pituitary-gonad axis and reproductive behavior between the sexes. Sex-changing fishes provide a unique opportunity to study mechanisms underlying sexual differentiation of the POA. In anemonefish (clownfish), which change sex from male to female, females have approximately twice the number of medium-sized cells in the anterior POA compared to males. This sex difference transitions from male-like to female-like during sex change. However, it is not known how this sex difference in POA cell number is established. This study tests the hypothesis that new cell addition plays a role. We initiated adult male-to-female sex change in 30 anemonefish (*Amphiprion ocellaris*) and administered BrdU to label new cells added to the POA at regular intervals throughout sex change. Sex-changing fish added more new cells to the anterior POA than non-changing fish, supporting the hypothesis. The observed effects could be accounted for by differences in POA volume, but they are also consistent with a steady trickle of new cells being gradually accumulated in the anterior POA before vitellogenic oocytes develop in the gonads. These results provide insight into the unique characteristics of protandrous sex change in anemonefish relative to other modes of sex change, and support the potential for future research in sex-changing fishes to provide a richer understanding of the mechanisms for sexual differentiation of the brain.

1. Introduction

Sex differences in the nervous system are essential for neuroendocrine regulation of gonadal physiology and sexual reproduction. The preoptic area of the hypothalamus (POA) is the hypothalamic node of the hypothalamic-pituitary–gonadal axis and an essential regulator of sexual behaviors and other social behaviors (Burbridge et al., 2016; Kanda, 2019; McCarthy, 2020; O'Connell and Hofmann, 2012, 2011). Sex differences in POA volume, cell number, or cell density are among the most widely-reported brain sex differences across all major vertebrate classes. They have been described in many mammals (Bleier et al., 1982; Bloch and Gorski, 1988; Clarkson and Herbison, 2006; Gorski et al., 1978; Madeira et al., 1999; Mohr et al., 2016; Roselli et al., 2004; Sickel and McCarthy, 2000) including humans (Allen et al., 1989; Byne et al., 2000; Garcia-Falgueras and Swaab, 2008; LeVay, 1991; Swaab and Fliers, 1985), in birds and reptiles (Crews et al., 1990; Hillsman et al., 2007; Panzica et al., 1991; Shevchouk et al., 2019; Viglietti-Panzica et al., 1986), in amphibians (Boyd et al., 1992; Moore et al., 2000; Takami and Urano, 1984), and in fishes (Okubo et al., 2019; Page et al., 2010; Yamashita et al., 2021).

Many of these sex differences have been linked to sex-specific reproductive physiology or behavior, and many of these sex differences are established by the actions of sex steroids during some critical period (Balthazart et al., 2009; Davis et al., 1996; Hillsman et al., 2007; McCarthy, 2020, 2008; Mohr et al., 2016; Panzica et al., 1991; Shevchouk et al., 2019). For example, in male rodents testosterone is secreted from the gonads during the perinatal period and is aromatized in the brain into estradiol to affect sexually-differentiated rates of apoptosis and dendritic growth in the developing POA (McCarthy, 2020). In female rodents, the absence of gonadal hormone secretion in the perinatal period allows the POA to develop along a female trajectory. Later, at the time of puberty, the surge of gonadal steroid secretion in both males and

* Corresponding author at: Beckman Institute, 405 N Mathews Ave, Urbana, IL 61801, USA.
E-mail address: jrhodes@illinois.edu (J.S. Rhodes).

https://doi.org/10.1016/j.ygcen.2022.114185
Received 31 May 2022; Received in revised form 26 November 2022; Accepted 7 December 2022
Available online 9 December 2022

females promotes cell proliferation and the gradual addition of new neurons and glia to sexually-differentiated regions of the POA throughout the peripubertal period, further differentiating those regions (Ahmed et al., 2008; Juraska et al., 2013; Mohr et al., 2017, 2016). The female-biased population in the anteroventral periventricular nucleus (AVPV) is essential in regulating the female ovulatory surge, while the male-biased population in the medial POA may regulate some aspects of male sexual behavior (Balthazart and Ball, 2007; McCarthy, 2020). Inhibiting peripubertal new cell addition to the female AVPV inhibits the activation of the female ovulatory surge (Mohr et al., 2017). Modulation of new cell addition to the POA is therefore an important mode of sexual differentiation, particularly for the female POA. A better understanding of its underlying mechanisms will be essential to more fully grasping female brain development and how it relates to reproductive health and dysfunction.

The deeply-conserved nature of the POA makes it an ideal focus for the comparative study of sexual differentiation in the brain (Kanda, 2019; O'Connell and Hofmann, 2012). Sex-changing fishes provide a particularly unique biological context to study sexual differentiation (Casas and Saborido-Rey, 2021; Erisman et al., 2013; Kuwamura et al., 2020; Lamm et al., 2015; Prim et al., 2022). In these fishes, sex is determined by social status in adulthood, meaning that a fish will spend part of its adult life as one sex (e.g., a male with functional testes and male-like POA), then if/when the fish ascends to the dominant position in the social hierarchy, it will change to the other sex (e.g., become female, replacing functional testes with ovaries and developing a female-like POA). Different species exhibit either protogynous (female-to-male), protandrous (male-to-female), or bidirectional sex change. Throughout sex change, circulating levels of gonad-derived androgens and estrogens generally fall between the more extreme values typical of reproductive males (high androgen and low estrogen) or females (low androgen and high estrogen) (Godwin and Thomas, 1993; Nakamura et al., 1989; Parker et al., 2022), and in some species the hormones have been shown to gradually shift over the course of sex change (Nakamura et al., 1989). Sex differences in cell numbers in the POA have been described in a number of sex-changing fishes, including protandrous anemonefish (*Amphiprion* spp.) (Dodd et al., 2019; Elofsson et al., 1997), the protogynous Bluehead wrasse (*Thalassoma bifasciatum*) (Grober et al., 1991; Grober and Bass, 1991), and bidirectional sex-changing gobies (*Lythrypnus dalli, Trimma okinawae*) (Black et al., 2004; Grober and Sunobe, 1996; Reavis and Grober, 1999). These POA sex differences appear to gradually reverse over the course of sex change, which takes weeks to months depending on the species (Black et al., 2004; Dodd et al., 2019; Grober et al., 1991). However, little is known about the mechanisms underlying this process, nor how the development of those POA sex differences may relate to the unique landscape of circulating gonad-derived steroids observed during sex change (Nakamura et al., 1989; Parker et al., 2022).

There is evidence supporting both gonad-dependent and gonad-independent modes of brain sexual differentiation in sex-changing fishes. Gonad-dependent modes are generally driven by gonad-derived androgens or estrogens, while gonad-independent modes may result from brain-derived steroids or other non-steroidal mechanisms. In the protogynous Bluehead wrasse, for example, there is evidence that a sex difference in gonadotropin-releasing hormone (GnRH) neuron number in the POA is controlled by gonadal androgens (Grober et al., 1991; Grober and Bass, 1991). As androgen secretion gradually increases over the course of female-to-male sex change in these fish, GnRH neuron number increases as well (Grober et al., 1991; Nakamura et al., 1989). This fits well with the broader consensus framework that has developed around sexual differentiation of the teleost brain generally, which holds that brain sexual differentiation is principally accomplished by gonadal steroids secreted in adulthood and not during early development (Okubo et al., 2019; Page et al., 2010). However, gonadal steroids do not control all aspects of brain sexual differentiation in sex-changing fish. For example, vasotocin neuron soma size and vasotocin gene expression in the POA is differentiated through gonad-independent mechanisms in the Bluehead wrasse (Semsar and Godwin, 2004, 2003). Recent work in the common clownfish *Amphiprion ocellaris*, which changes sex from male-to-female, identified a sex difference in the POA that is differentiated independent of gonadal influence as well (Dodd et al., 2019). Female anemonefish had approximately twice the number of medium-sized cells in the anterior POA compared to males, and this sex difference emerged within six months of sex change, before gonadal sex change is complete (Dodd et al., 2019). The phenotype and function of these cells is yet to be established, but their number and distribution throughout the anterior POA is most consistent with a neuronal identity.

One hypothesis to explain the doubling of this cell population before gonadal sex change is complete in anemonefish is that a period of enhanced cell proliferation, survival, or differentiation of new cells is triggered during sex change by gonad-independent means. Cellular proliferation (and subsequent differentiation into neurons) is known to persist throughout the fish brain throughout adulthood, including in the POA (Ganz and Brand, 2016; Kaslin et al., 2008; Page et al., 2010; Pellegrini et al., 2016; Zupanc, 2021). Radial glia that line the ventricle within the POA, and throughout the adult fish brain, serve as an asymmetrically-dividing progenitor cell pool as well as a scaffolding for neuroblast migration into the brain parenchyma (Ganz and Brand, 2016; Pellegrini et al., 2016; Zupanc, 2021). Continuous cell proliferation and neurogenesis allows for continuous growth throughout life in fish, as well as central nervous system repair after injury (Diotel et al., 2013; Ganz and Brand, 2016; Kaslin et al., 2008). Estrogens and androgens are known to play a role in regulating proliferation and survival of new neurons in the forebrain and hypothalamus (Diotel et al., 2013; Narita et al., 2018; Pellegrini et al., 2016). The estrogens in question appear to be brain-derived (Diotel et al., 2013), whereas androgen effects have only been demonstrated through exogenous administration (Narita et al., 2018). Rates of cell proliferation in the POA are also sensitive to social status in some teleost fish (Maruska et al., 2012). It is plausible that similar mechanisms may also be employed in the context of socially-controlled sex change. Despite all of this, no research has yet tested the potential role for new cell addition to contribute to brain sex change in any sex-changing fish.

In the present study we aimed to test whether a period of increased new cell addition to the POA could be identified that may contribute to the emergence of a sex difference in POA cell number during sex change in anemonefish. We use the term "cell addition" to refer to the combined process of cell proliferation, survival, migration and differentiation, ultimately resulting in a net increase in the number of new cells. The sizeable sex difference observed in the anemonefish POA appears reliably within six months, providing a good opportunity to study the remodeling of the POA over a predictable timeframe (Dodd et al., 2019). We quantified the number of new cells added to the POA at regular intervals within six months after the onset of sex change in *Amphiprion ocellaris*, with the goal of identifying a time period within the six month interval where more new cells were added to the POA in sex-changing fish.

2. Methods

2.1. Animals and husbandry

Fish were bred in-house from fish originally obtained from Ocean Reefs and Aquariums (Fort Pierce, FL). A total of 60 male *Amphiprion ocellaris* were used in this study to generate sex-changing pairs (see below, "2.2. Initiation of Sex Change"). Anemonefish are among the best-studied protandrous species, in part due to their unique life history (Casas et al., 2022). They live in small groups of approximately 2–4, sometimes more, around an anemone host. The group consists of one dominant female and her breeding male partner (the next fish in the hierarchy), often along with one or a few smaller non-breeding fish. If the dominant female disappears, the breeding male will change sex to

become female and the largest non-breeder will mature into a reproductive male. Sex change can also be initiated in the laboratory by pairing two males together in a tank (Dodd et al., 2019). The male to establish dominance will change sex. Anemonefish sex change is typified by an individually-variable timecourse, spanning two to six months or more (Casas et al., 2016; Dodd et al., 2019; Fricke and Fricke, 1977; Godwin, 1994; Moyer and Nakazono, 1978). This is the case in the laboratory as well as in the wild, although laboratory sex change generally does appear to proceed more slowly. Over the course of sex change the dominant fish gradually loses testicular tissue (Casas et al., 2016; Dodd et al., 2019; Godwin, 1994). And in cases where sex change is initiated through male-male pairing, both the dominant as well as subordinate fish gradually lose testicular tissue at a comparable rate (Dodd et al., 2019). This is hypothesized to reflect a state of reproductive stasis that both fish occupy while the process of sex change unfolds in the dominant fish (Dodd et al., 2019; Fricke, 1983).

Prior to the experiment, each of the males were paired with females and spawning regularly. Another four breeding pairs (4 males and 4 females) were used as controls. Each tank contained one terra-cotta pot (diameter 6″) as a nest site and spawning substrate. All fish used in this study were reproductively mature, having been observed spawning with fertilized eggs at least once within three months preceding the experiment. Breeding pairs in our colony are continuously spawning and nests are checked for the presence of fertilized eggs daily. Fish were housed in twenty-gallon tall (24″ x 12″ x 16″) aquarium tanks integrated with a central circulating filtration system. Conditions mimicked the *A. ocellaris* natural environment (system water temperature range 26–28C, pH range of 8.0–8.4, specific gravity of 1.026, 12:12 photoperiod with lights on at 0700 and off at 1900 h). Fish were fed twice daily with Reef Nutrition TDO Chroma Boost pellets. Experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

The fish used in this study were also involved in a related study with the goal of investigating the timecourse of behavioral and hormonal sex change, the results of which are now published (Parker et al., 2022). In the three days prior to the day of euthanasia and tissue collection, all fish were subjected to behavioral assays consisting of two fifteen-minute encounters with a novel male fish, two fifteen-minute encounters with a novel female fish, and a thirty-minute encounter with a clutch of unrelated eggs. The reader is directed to that published report for a complete description of the behavioral assays (Parker et al., 2022). Relevant portions of the methods and results from that report are reiterated and discussed in the present report.

2.2. Initiation of sex change

Sex change was initiated by pairing two male fish together in a tank, as in previous work (Dodd et al., 2019). When two male *A. ocellaris* are paired together in a tank they will display aggressive interactions to establish dominance (Yaeger et al., 2014). Generally, the larger fish will establish dominance and as a result change sex to female. To standardize the dominance contest we paired fish based on body size, keeping the body size discrepancy as consistent as possible across pairs. On average the larger fish in each pair was 15 ± 3 % longer (mean ± standard deviation) and 51 ± 18 % more massive than its partner.

Dominance status in anemonefish is readily observable to an experimenter. Fish establish and maintain their dominance through aggressive displays (chasing, biting, lunging at subordinate fish). Subordinate fish do not aggress in response, and instead display submissive behaviors (e.g., fleeing when chased, trembling) (DeAngelis et al., 2020; Fricke and Fricke, 1977; Gonzalez et al., 2021; Iwata and Manbo, 2013; Wong et al., 2013). Newly established pairs were monitored daily by experienced researchers (CGP and JSR) during the first week after pairing to qualitatively assess the direction of aggressive and submissive behaviors within pairs, as in previous work (Dodd et al., 2019). Based on this assessment we confirmed that the larger fish was the one to establish dominance in all pairs. Dominant fish are also expected to grow substantially to cement their dominant status and attain female size, which is approximately 25 % longer and 300 % more massive than the male partner (Buston, 2003; Fricke, 1979). To quantify change in body length and body mass over time we collected body length and body mass measurements at the time of pairing and again at the time of behavior and blood collection. Changes in body length and body mass were then calculated for each fish as percent change from initial length and mass. Indeed, we found that dominant fish in this study grew at a greater rate than subordinate fish, consistent with dominance establishment and the initiation of change (Parker et al., 2022).

Sixty male *A. ocellaris* were removed from their female mates and paired off to create thirty dominant-subordinate pairs. This generated n = 30 dominant sex-changing fish and n = 30 subordinate non-changing fish. Four mature male–female breeding pairs were chosen from our colony to serve as controls (n = 4 control males and n = 4 control females). These fish had been observed spawning and rearing viable eggs at least once within three months prior to the study. Control pairs remained in their home tanks, receiving experimental treatments identical to the newly established experimental pairs.

2.3. Terms and definitions regarding sex change

Within the context of this report we will define sex and sex change as follows. Male and female fish are reproductively mature, having successfully fertilized or laid eggs, respectively, within the past three months. Control male and female fish used in this study satisfied these criteria. When a male fish, in the absence of a female, establishes dominance over a subordinate tankmate, the dominant fish is no longer considered to be male and is instead considered to be in the process of sex change. The partners of these dominant sex-changing fish are considered to be subordinate non-changing fish. Subordinate non-changing fish are distinct from typical male fish because of their unique social context, as well as evidence that subordinate non-changing fish enter a period of reproductive dormancy characterized by regression of testicular tissue in the gonads (Dodd et al., 2019).

It is generally accepted that the process of sex change in anemonefish is complete when a fish has gonads that are completely comprised of ovarian tissue, including bearing eggs at various stages of vitellogenesis, and lacking testicular tissue (Dodd et al., 2019; Fricke and Fricke, 1977; Godwin, 1994). To our knowledge, all available evidence indicates that vitellogenic oocytes rarely co-occur with any amount of testicular tissue remaining, and by the time a new female fish has spawned for the first time it has lost all testicular tissue. Based on these criteria, we happened to find 2 out of 29 dominant fish in this study (7 % of dominant fish) had completed gonadal sex change by the time of tissue collection. One of these fish was in the 18 week timepoint, and one was in the 26 week timepoint (see below, “2.4. BrdU Administration”, for information on timepoints). To distinguish these fish from control females we will refer to these fish as “new females”, and we refer to the tankmate of a new female as a “new male” to distinguish it from a control male. Neither of these sex-changed fish had laid eggs before tissue collection.

Terms, as defined above, will be abbreviated as follows in the text and figures: male fish (M), female fish (F), dominant sex-changing fish (D), subordinate non-changing fish (S), new female (NF), new male (NM).

2.4. BrdU administration

Each of the 30 dominant-subordinate pairs were assigned to one of six timepoints for bromodeoxyuridine (BrdU) dosing (n = 5 pairs each timepoint). BrdU is a thymidine analogue that is incorporated into the DNA of dividing cells. Immunohistochemical detection of BrdU in a cell indicates that the cell was born at the time of BrdU administration. Fig. 1 summarizes the BrdU dosing and tissue collection schedule. BrdU was administered to fish for five consecutive days beginning either 2, 6, 10,

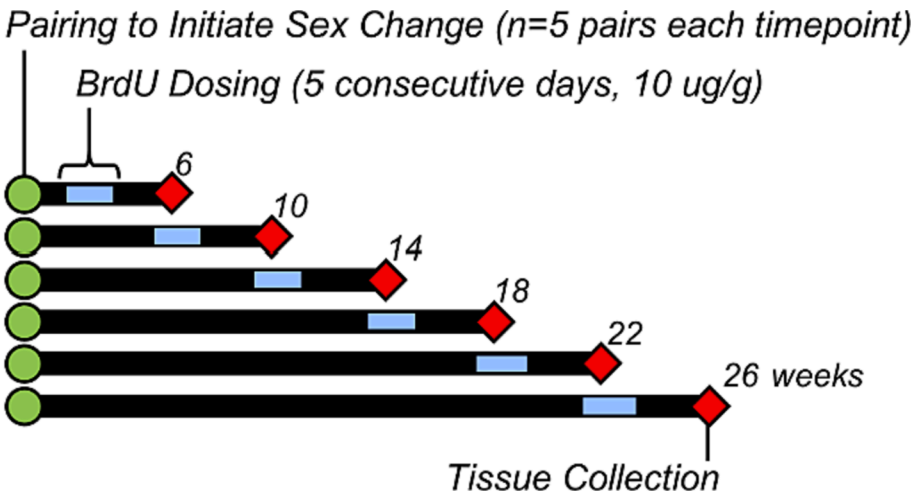


Fig. 1. Summary of study design. Thirty dominant-subordinate pairs were generated from sixty male *A. ocellaris*. Pairs were assigned to one of six timepoints for brain and gonad tissue collection (n = 5 pairs per timepoint). Timepoints were scheduled for tissue collection at 6, 10, 14, 18, 22, and 26 weeks after pairing to initiate sex change. BrdU was administered to fish over five consecutive days, with the last day of BrdU dosing falling exactly twenty-eight days (four weeks) before tissue collection. The four week incubation period between BrdU dosing and tissue collection allows time for newly-proliferated cells to either migrate and differentiate or turn over.

14, 18, or 22 weeks after pairs were established (n = 5 pairs each timepoint). Fish were then left undisturbed for 4 weeks to allow BrdU-labeled cells to proliferate, migrate and differentiate, then fish were euthanized and tissue was collected. Thus, the timepoints for tissue collection were at 6, 10, 14, 18, 22, or 26 weeks, respectively. Groups will be referred to by these tissue collection timepoints throughout the report and figures.

BrdU was administered to all fish during their designated dosing period for 5 consecutive days between 1000 and 1200 h each day, with the last dose being administered exactly 28 days (4 weeks) prior to the day of euthanasia and tissue collection. BrdU was prepared in a sterile saline solution (0.9 %) and injected intraperitoneally at a final dosage of 10 ug/g body mass. Unfortunately, one pair of fish assigned to the week 18 timepoint had to be removed from the study because the dominant fish died after BrdU dosing. This, along with the assignment of the two fish that completely changed sex into a separate group for analysis (see above, "2.3. Terms and Definitions Regarding Sex Change"), left n = 3 dominant-subordinate pairs at the 18 week timepoint, n = 4 pairs at the 26 week timepoint, and n = 5 pairs at all other timepoints. The maximum time of 26 weeks (six months) was chosen because our previous study found that the sexually-differentiated anterior POA cell population is reliably female-like in cell number within six months of sex change (Dodd et al., 2019).

2.5. BrdU Immunohistochemistry and quantification

Following euthanasia, brains were extracted and placed in a chilled 4 % paraformaldehyde solution (pH 7.4) for 24 h. Brains were then treated in 30 % sucrose solution at 4 °C for 24 h, then embedded in OCT medium and frozen at −80 °C until sectioning. Brains were sectioned on a cryostat in the coronal plane at 18 um and mounted directly onto slides (SuperFrost Plus, Fisher Scientific) in two series. One series was used in the present study. After sectioning, slides were allowed to dry for 24 h at room temp and then stored in slide boxes at −80 °C until immunohistochemical processing.

Immunohistochemical staining for BrdU+ cells was carried out as follows. First, slides were removed from −80 °C storage to defrost and dry at room temp for 24 h. Slides were then immersed in a chilled bath of 4 % paraformaldehyde solution for 10 min, then washed in Tris-buffered saline (TBS) 3 times, 5 min each wash. Then, to reduce endogenous peroxidase activity, slides were immersed in 0.6 % H_2O_2 solution for 30 min, followed by another 3 × 5 min TBS washes. For antigen retrieval slides were then incubated in a 50 % formamide solution at 65 °C for 90 min, then washed in 2 M saline sodium citrate at room temp for 15 min, incubated in 2 M HCl at 37 °C for 30 min, then in 0.1 M borate buffer (pH 8.5) at room temp for 10 min, then finally slides were washed in TBS 3 × 5 min. Next, a hydrophobic barrier was drawn around the boundary of the slide surface with an ImmEdge PAP Pen (Vector Labs). A blocking solution (3 % normal goat serum and 0.1 % Triton-X in TBS) was then pipetted onto the slides for an incubation at room temp in a humid chamber for 1 h. Slides were then incubated in the primary antibody solution (rat monoclonal anti-BrdU at 1:1000 in blocking solution; Abcam #ab6326) at 4 °C in a humid chamber overnight (approximately 18 h). Following the primary antibody treatment, slides were washed again in TBS 3 × 5 min then incubated in the secondary antibody solution (goat anti-rat, biotinylated, at 1:250 in blocking solution; Vector Labs #BA-9400–1.5) at room temp in a humid chamber for 2 h. Slides were washed in TBS 3 × 5 min, then were incubated in an avidin–biotin solution (Vectastain ABC Kit, Vector Labs) at room temp in a humid chamber for 1 h, followed by another 3 × 5 min washes in TBS. Slides were then treated in a 3,3′-diaminobenzidine solution (SigmaFast DAB Kit, Sigma Aldrich #D4418), then briefly washed in TBS 3 × 1 min. Finally, slides were dehydrated, counterstained, and coverslipped as follows: TBS for 5 min, 1 % periodic acid for 5 min, methylene blue-azure II solution for 7 min, brief dunk in 95 % ethanol, brief dunk in 100 % ethanol, treat in 100 % ethanol for 1 min, another fresh 100 % ethanol for 3–5 min (depending on stain intensity), xylenes for 10 min, coverslip using Permount mounting medium (Fisher Scientific).

Brains were imaged to quantify number of BrdU+ cells and volume of the POA at 100x total magnification on a Zeiss AxioScope A1 light microscope using AxioVision software (Zeiss). The POA was identified and outlined in images with reference to a high-quality brain atlas for the cichlid fish *Astatotilapia burtoni* created and maintained by Karen Maruska's Lab, as well as other published atlases for *A. burtoni* (Fernald and Shelton, 1985). Based on these references and following similar past work (Dodd et al., 2019) we split the POA into anterior, middle, and posterior subdivisions (aPOA, mPOA, and pPOA respectively). Moving through the brain from anterior to posterior, the beginning of the aPOA coincides with the middle of the anterior commissure. The boundary between aPOA and mPOA is defined by the appearance of the nearby entopeduncular nucleus, and the boundary between the mPOA and pPOA is defined by the end of the entopeduncular nucleus and the lateral extension of the ventral part of the POA along the optic nerve. The end of the pPOA coincides with the start of the nearby anterior thalamic nucleus. These POA divisions follow previous work (Dodd et al., 2019), and result in the following relationships with POA divisions of the cichlid fish brain: the aPOA consists entirely of anterior parvocellular POA, the mPOA consists largely of magnocellular POA, and the pPOA consists of gigantocellular and posterior parvocellular POA. Previously, we found an increase in cell number specifically in the aPOA during sex change using a Nissl stain and counting total number of cells in each POA subdivision (Dodd et al., 2019). In the present work, BrdU+

immunostained cells within the boundaries of the POA were manually counted for each section using ImageJ. Immunohistochemistry targeting BrdU stains the nuclei of the cell. A stained object had to satisfy-three criteria to be counted as a BrdU+ cell: shape (must be approximately circular or elliptical in shape, as is expected of neuronal nuclei), size (approximately 4–8 um in diameter, as is expected of neuronal nuclei in our fish), and stain quality (must clearly stand out against the background and have a clear edge). Examples of BrdU+ cells meeting these criteria appear in Fig. 4. An estimate of total cell count for each subdivision was then generated by calculating the average BrdU+ cells per section for each subdivision, multiplying this value by the total number of sections in the subdivision (including missing or damaged sections from which no counts could be taken), then multiplying this value by two to account for working from one of two series of brain sections. The volume of the POA in each section was also quantified and used to calculate an estimated total volume for each POA subdivision in a similar manner. Density of BrdU+ cells in each POA subdivision was also calculated as total cell count divided by volume. All BrdU+ cell counts and POA volume estimates were collected by a single researcher following the *a priori* agreed upon set of criteria outlined above.

2.6. Gonadal histology and quantification

Gonads were collected and their composition assessed to determine the extent of gonadal sex change relative to brain sex change. Following euthanasia, the fish bodies were placed in chilled 4 % paraformaldehyde solution (pH 7.4) for 24 h, then treated in 30 % sucrose solution at 4 °C for 24 to 48 h. Bodies were then trimmed to remove excess tissue leaving only the abdominal cavity containing the gonads to be embedded in OCT cryosectioning medium (Fisher Scientific) and frozen at −80 °C until sectioning. Tissue was sectioned at 40 um in the sagittal plane on a cryostat and mounted directly onto slides (SuperFrost Plus, Fisher Scientific) in a single series. After sectioning, slides were allowed to dry at room temp for 24 h and then were stored in slide boxes in −80 °C until staining.

Gonad sections were stained with hematoxylin and eosin (H&E) as follows. First, slides were removed from −80 °C storage to defrost and dry at room temp for 24 h, then they were immersed in a chilled bath of 4 % paraformaldehyde solution for 10 min, then briefly washed in distilled water. Slides were then immersed in hematoxylin solution (Sigma Aldrich #HHS-32) for 6 min, then washed in gently running tap water for 5 min. This was followed by an acid alcohol solution (0.2 M HCl in 70 % ethanol), a wash in running tap water for 1 min, a lithium carbonate solution (0.2 M lithium carbonate in water), and another wash in running tap water for 5 min. Slides were then rinsed in a series of 95 % ethanol baths (5 clean baths, 1–3 dips each depending on stain intensity), then immersed in an eosin-phloxine B solution (1 % eosin Y and 0.1 % phloxine B in 74 % ethanol with 0.4 % glacial acetic acid) for 1 min. Slides were then dehydrated and coverslipped as follows: 95 % ethanol for 3 min, another 95 % ethanol for 3 min, 100 % ethanol for 3 min, another 100 % ethanol for 3 min, xylenes for 10 min, then coverslipped with Permount mounting medium (Fisher Scientific).

Gonads were imaged and gonadal composition was quantified following established procedures (Dodd et al., 2019; Gonzalez et al., 2021). First, to estimate the proportions of major tissue types in the gonad for each fish (% testicular tissue, % non-vitellogenic ovarian tissue, and % vitellogenic ovarian tissue), twenty gonad sections were sampled at random for each fish and imaged at 100x total magnification on a Zeiss AxioScope A1 light microscope using AxioVision software (Zeiss). Using Adobe Photoshop CS6, the gonad was outlined in the image and the Quick Selection tool was used to differentiate testicular tissue from ovarian tissue automatically with manual correction, following established procedures (Dodd et al., 2019; Gonzalez et al., 2021). The area proportion of the gonad covered by each of these tissue types (% testicular, % non-vitellogenic ovarian, % vitellogenic ovarian) was calculated for each section. These proportions were then averaged over the twenty sections to produce an estimated proportion for each tissue type for each fish. Then, to estimate the total gonad volume for each fish, half of all sections were selected at random and imaged at 25x total magnification on a Zeiss AxioScope A1 light microscope using AxioVision software. For each image the gonad was outlined using Adobe Photoshop CS6 and the area was recorded. The average area per section was then calculated for each fish, then this value was multiplied by the total number of sections through the gonad and the thickness of each section (40 um) to calculate an estimate of the total gonad volume. This was then used to calculate a normalized gonad volume value (akin to a gonadosomatic index) for each fish as total gonad volume divided by body mass.

2.7. Statistical analysis

Statistical analyses were conducted using R (4.1.0) and SAS (SAS Institute, Cary, NC). P-value < 0.05 was considered statistically significant for all analyses. To test whether dominant sex-changing fish and their subordinate partners differed between one another over time in terms of gonad composition or gonad size, we fit linear mixed-effect models for testicular tissue proportion and for normalized gonad volume. Status (dominant vs subordinate) was entered as a fixed categorical factor, timepoint as a fixed continuous factor, and tank as a random categorical factor. The random factor tank accounts for possible inter-tank differences as well as possible variation due to BrdU solution batch differences, as both fish in each tank were injected at the same time from the same batch. Timepoint was treated as a continuous factor to model a predicted linear decline in testicular tissue proportion, as observed in previous work (Dodd et al., 2019). Analyses were repeated with timepoint as a categorical factor as well yielding no additional insight (see Results below). Then, to test how dominant and subordinate fish compared to control males and new males in terms of testicular tissue proportion and normalized gonad volume, we collapsed dominant and subordinate fish across timepoints and fit a mixed-effects model for each outcome measure followed by Fisher's LSD post-hoc when appropriate. For these analyses the group factor had four levels (dominant, subordinate, new male, male), and tank was included as a random factor. Control females and new females were omitted from these analyses for two reasons. First, they have zero testicular tissue by definition, while all other groups have non-zero testicular tissue by definition, making statistical comparisons on the basis of testicular tissue moot. Second, tissue was collected at random timepoints in the reproductive cycle for each female, and total gonad volume is expected to vary widely over the course of the cycle as eggs develop.

To test whether there was a timepoint(s) at which dominant sex-changing fish accumulated significantly more new BrdU+ cells than their subordinate non-changing partners, we fit linear mixed-effects models similar to those fit above for gonad measurements over time, with the exception that timepoint was treated as a categorical factor instead of continuous. This is because we did not necessarily predict a linear increase or decrease in BrdU+ cells over time in sex-changing fish. Then, to test how dominant and subordinate fish compared to all other groups, we collapsed them across timepoints as above and fit a mixed effects model with status as a fixed factor and tank as a random factor. Separate models were fit for BrdU+ cell count in each POA subdivision (aPOA, mPOA, pPOA). Subsequently, similar models were fit with the addition of a covariate to account for individual differences in brain or POA size. Models for BrdU+ cell count for each subdivision were fit with brain mass as a covariate, then again with POA subdivision volume as a covariate. Subdivision volume, when used as a covariate or denominator for density calculation, has the advantage of being more proximately related to POA BrdU+ cell count than whole brain mass. However, the disadvantage is that subregion volume estimation is subjective in nature and precise subregion boundaries are not as well defined in non-model species like *A. ocellaris* as they are in mainstream study systems. This naturally limits the maximum possible accuracy of volume estimates,

making volume a potential source of unwanted noise. Brain mass has the advantage of being objective and straightforward to collect, but it is much less proximate to BrdU+ cell count in a particular subdivision of the POA. Models were also fit for BrdU+ cell density in each subdivision.

Normality of residuals was assessed based on diagnostic plots (Q-Q plot and histogram of residuals) and skewness and kurtosis (skewness between −1 and 1, kurtosis between −2 and 2). Homogeneity of variance was checked using Levene's test. Based on these metrics we determined that no transformations were necessary before analysis.

3. Results

3.1. Gonad Composition. Sex-changing fish and their non-changing partners lost testicular tissue in unison

All major gonadal tissue types (testicular, non-vitellogenic ovarian, vitellogenic ovarian) were identified in gonad sections (see Fig. 2. for representative gonad images from fish of each group in this study). No effect of time ($p = 0.18$), status ($p = 0.71$), or interaction between time and status ($p = 0.90$) was observed when time was entered as a continuous variable for testicular tissue proportion (Fig. 3A). Nor was an effect of time ($p = 0.90$), status ($p = 0.44$), or an interaction ($p = 0.40$) observed for normalized gonad volume (Fig. 3B). Repeating these analyses with timepoint as a categorical factor returned no significant effect of time ($p = 0.23$) or status ($p = 0.96$), a significant interaction between status and timepoint for testicular tissue proportion ($F_{5,42} = 2.86$, $p = 0.04$), and no significant effect of time ($p = 0.90$), status ($p = 0.85$), or an interaction ($p = 0.49$) for normalized gonad volume. Post hoc analysis on testicular tissue proportion revealed five significant pairwise differences, four of which showed decreased proportion at a later time-point compared to an earlier one. These were between dominant fish at week 14 and 22 ($p = 0.02$), subordinate fish at week 10 and 14 ($p = 0.01$), dominant fish at week 14 and subordinate fish at week 26 ($p = 0.04$), and subordinate fish at week 10 and dominant fish at week 22 ($p = 0.03$). The fifth significant pairwise difference detected was a greater proportion in dominant compared to subordinate fish at week 14 ($p = 0.007$).

Collapsing dominant and subordinate fish across timepoints and comparing them against control males and new males (Fig. 3C) revealed a significant effect of status on testicular tissue proportion ($F_{3,56} = 3.95$, $p = 0.01$), such that control males had significantly more testicular tissue than all other groups (all $p < 0.05$). There was no difference between any of the groups in terms of normalized gonad volume ($p = 0.44$) (Fig. 3D). Control and new females were omitted from testicular proportion analysis because their gonads, by definition, contain zero testicular tissue, and they were omitted from gonad volume analysis because female gonad volume is expected to vary widely across the reproductive cycle.

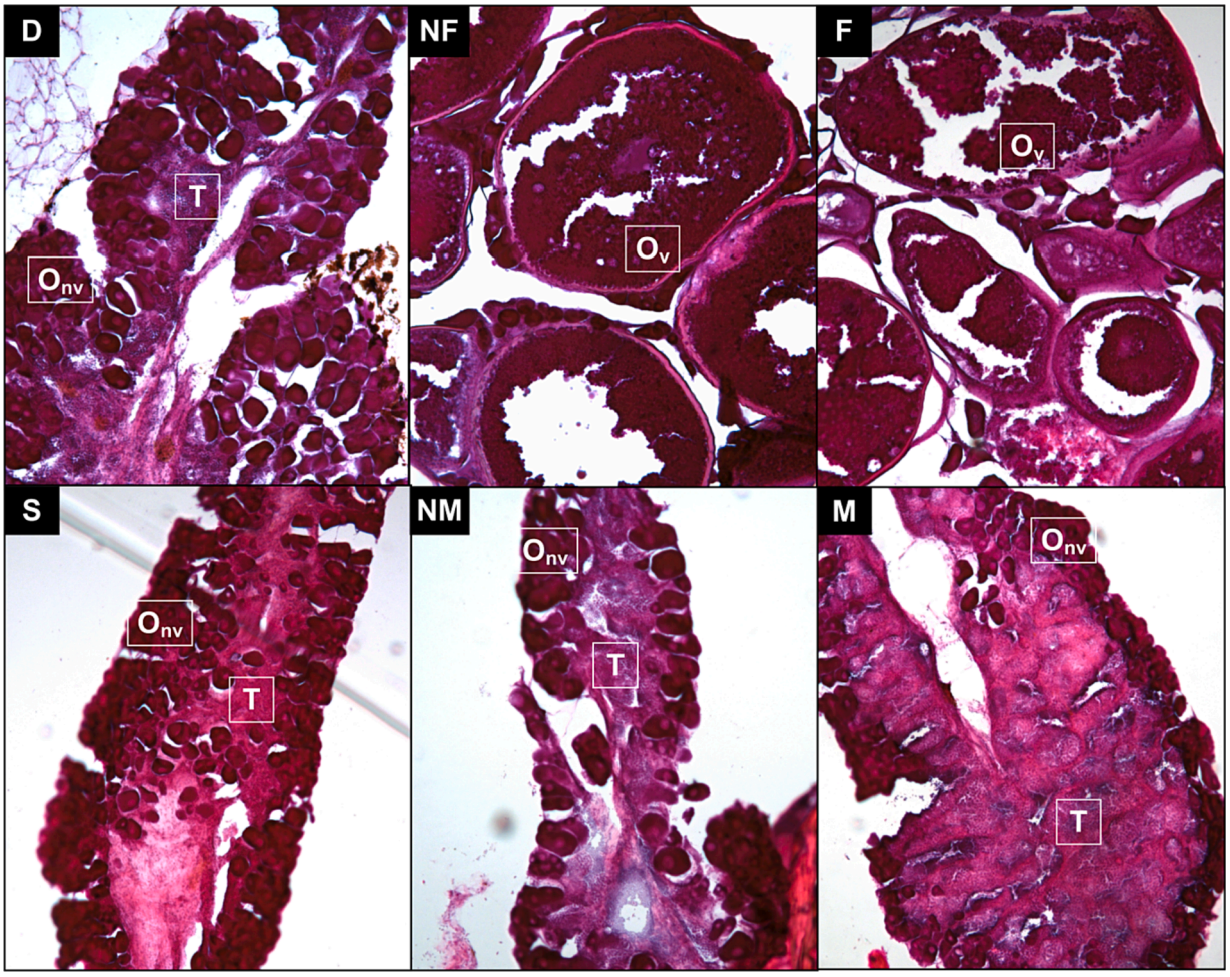


Fig. 2. Representative images of hematoxylin and eosin stained gonad sections. Each image is labeled for the group it belongs to (dominant sex-changing, D; subordinate non-changing partner, S; new female, NF; new male, NM; control female, F; control male, M), and major tissue types are identified (testicular tissue, T; non-vitellogenic ovarian tissue, O_{nv}; vitellogenic ovarian tissue, O_v). In non-female fish, testicular tissue forms the core of the gonad while ovarian tissue surrounds it. Note that gonads of dominant fish, subordinate fish, and new males are similar in composition, although the gonads of dominant fish appear slightly larger overall. Also note that new females and control females are similar, with many large developing vitellogenic oocytes.

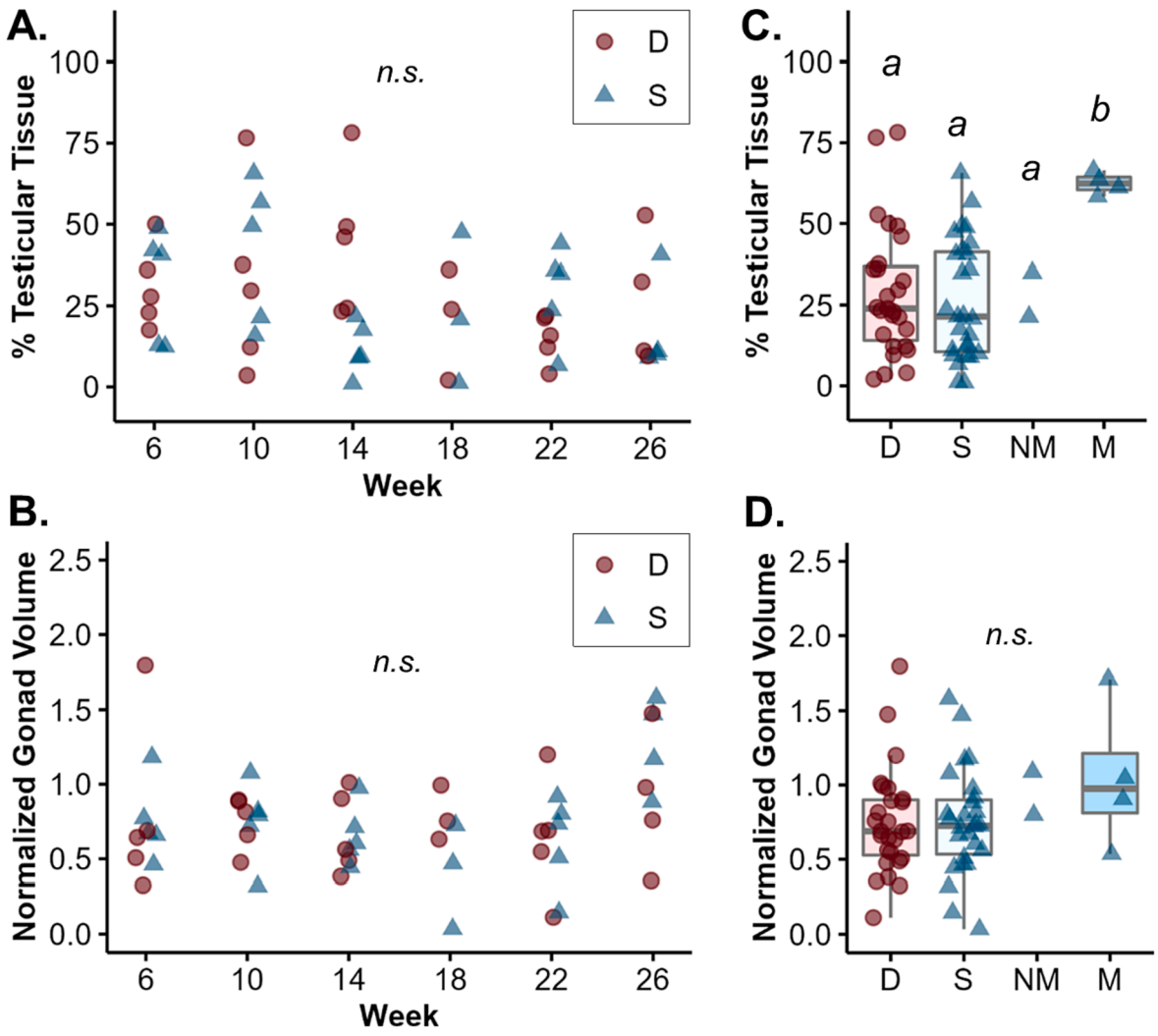


Fig. 3. Sex-changing fish and their non-changing partners lost testicular tissue in unison. A) Proportion of testicular tissue in the gonad did not differ between dominant sex-changing fish and subordinate non-changing fish over time, nor did B) gonad volume. C) Sex-changing fish had testicular tissue proportion comparable to their non-changing partners and new males. Control males had a higher testicular proportion than all other groups, and control females and new females had zero testicular tissue in their gonads (control females and new females not shown in figure). D) Gonad volume did not differ between any of the groups compared when collapsing across time. Control and new females are omitted from gonad volume analysis because tissue was collected at random timepoints in the reproductive cycle, and total gonad volume is expected to vary widely over the course of the cycle as eggs develop. Groups are abbreviated as follows: dominant sex-changing, D; subordinate non-changing partner, S; new male, NM; control male, M. Letters over boxplots distinguish groups that are significantly different from one another. Boxplots capture the inter-quartile range (IQR, first to third quartile), whiskers extend to the largest value within 1.5 IQR of the box, and median is marked by a horizontal line within the boxplot.

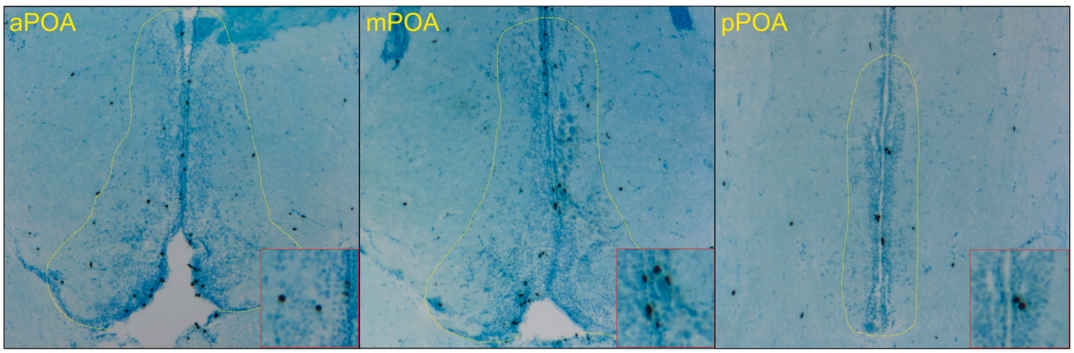


Fig. 4. BrdU+ cells were found throughout the POA. Representative images through the anterior, middle, and posterior POA (aPOA, mPOA, pPOA, respectively) immunostained to reveal BrdU and counterstained with methylene blue & azure II. In each image, the POA region is outlined in yellow. Insets show a magnified view of BrdU+ cells from each image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. POA BrdU+ Cell Count. Sex-changing fish accumulate more new cells in the aPOA than non-changing fish.

BrdU+ cells were found throughout all subdivisions of the POA (see Fig. 4 for representative images of BrdU+ cells in each POA subdivision). Previous research identified a female-biased sex difference in aPOA cell number that emerges within six months of sex change (Dodd et al., 2019). Based on this, we expected to observe more new cells added to the aPOA, but not the mPOA or pPOA, at one or more timepoints during sex change in dominant fish. Indeed, there was a main effect of status on BrdU+ cell count in the aPOA, such that dominant sex-changing fish had more new cells than their non-changing partners ($F_{1,20} = 10.34$, $p = 0.004$), and no main effect of time ($p = 0.83$) or interaction effect ($p = 0.85$) (Fig. 5A). No significant effect of time ($p = 0.68$), status ($p = 0.10$), or an interaction ($p = 0.38$) was detected in the mPOA (Fig. 5B), nor was an effect of time ($p = 0.57$), status ($p = 0.48$),or an interaction ($p = 0.34$) detected in the pPOA (Fig. 5C). Comparing dominant and subordinate fish collapsed across timepoints against all other groups revealed a significant effect of status on BrdU+ cell count in the aPOA ($F_{5,29} = 4.29$, $p = 0.005$) (Fig. 5D). Dominant fish again had a significantly greater BrdU+ cell count than subordinate fish ($p = 0.002$), and new female fish had a significantly greater count than all other groups (all $p < 0.05$) except for control males ($p = 0.16$). There was an effect of status in the mPOA as well ($F_{5,29} = 3.83$, $p = 0.009$) (Fig. 5E), and this effect was driven by new females which had significantly more BrdU+ cells than all other groups (all $p < 0.05$) except for dominant fish ($p = 0.07$). There was no effect of status in the pPOA ($p = 0.11$) (Fig. 5F).

These differences in BrdU+ cell count may be attributable, in part or in whole, to differences in POA size, as BrdU+ cell count is expected to scale with POA volume as in similar past work (Ahmed et al., 2008). To address this possibility, we fit the same models described above with the addition of either POA subdivision volume or whole brain mass as a covariate. We also tested models for BrdU+ cell density. None of these models detected any effect of time when comparing dominant and subordinate fish over time (all $p > 0.05$), so in the interest of clarity we are only describing the models collapsed across timepoints comparing all groups. The statistics and estimated marginal means ± standard error associated with the models for BrdU+ cell counts are summarized in Table 1, and the statistics and observed means ± standard error for subdivision volume and cell density are summarized in Table 2. With brain mass in the model as a covariate, there was still a significant main effect of status on BrdU+ cell count in the aPOA ($F_{5,28} = 3.60$, $p = 0.01$) and mPOA ($F_{5,28} = 3.61$, $p = 0.012$). However, the nature of this effect changed such that post hoc analysis no longer detected a significant pairwise difference between dominant and subordinate fish in the aPOA ($p = 0.80$). The effects driven by new females in the aPOA and mPOA largely remained when including brain mass as a covariate (see Table 1 for pairwise differences). Including subregion volume as a covariate caused the main effect of status on BrdU+ cell count to be lost in the aPOA ($p = 0.096$) but not the mPOA ($F_{5,28} = 3.29$, $p = 0.02$). The effect

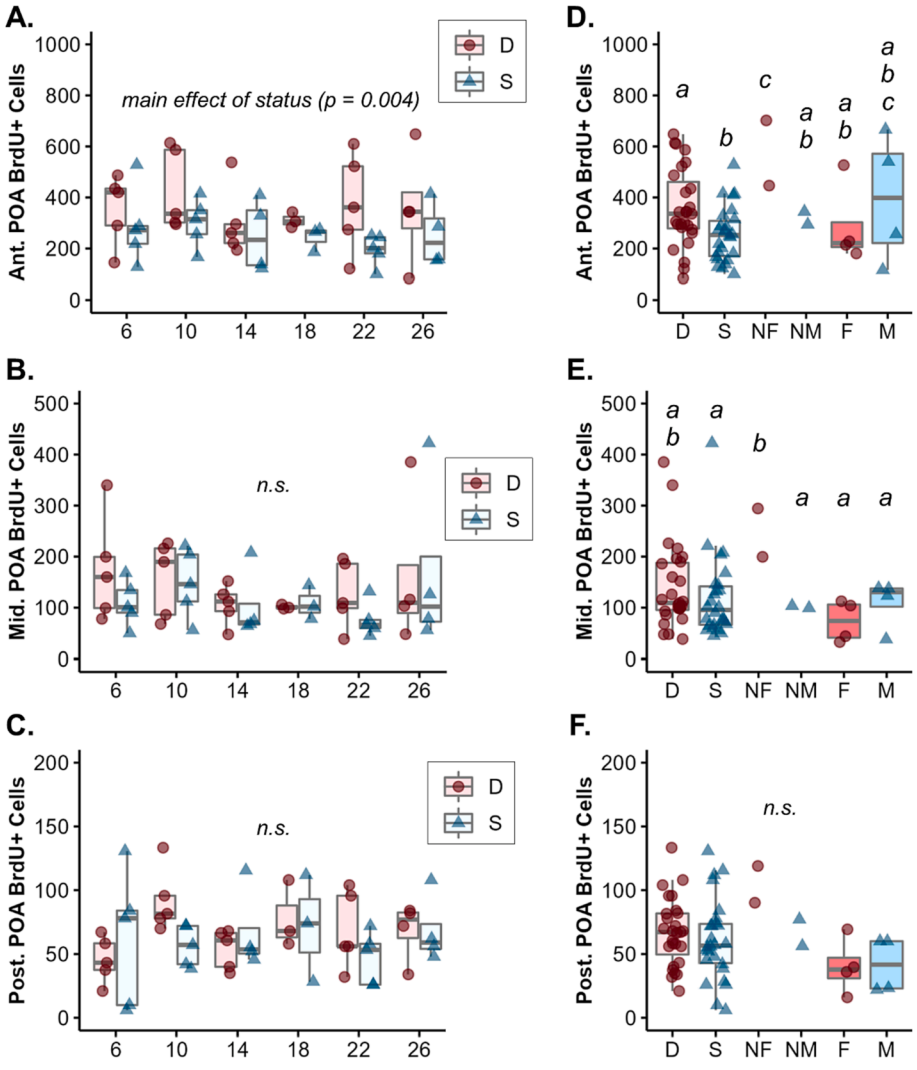


Fig. 5. Sex-changing fish accumulate more new cells in the aPOA during sex change. A) Dominant sex-changing fish gradually accumulated more new cells in the aPOA than non-changing fish in the six months following initiation of sex change. B) There was no effect of status or time on BrdU+ cells in the mPOA, C) nor were there any effects in the pPOA. D) In the aPOA new females accumulated even more BrdU+ cells than sex-changing fish, as well as all other groups except for control males, and E) in the mPOA new females had a greater number of BrdU+ cells than all other groups except for dominant fish. Note that there are only n = 2 new females, meaning these results regarding new females should be interpreted cautiously. F) None of the groups compared when collapsing dominant and subordinate fish across time differed in new cells in the pPOA. Groups are abbreviated as follows: dominant sex-changing, D; subordinate non-changing partner, S; new female, NF; new male, NM; control female, F; control male, M. Letters over boxplots distinguish groups that are significantly different from one another. Boxplots capture the inter-quartile range (IQR, first to third quartile), whiskers extend to the largest value within 1.5 IQR of the box, and median is marked by a horizontal line within the boxplot.

Table 1
Statistics and estimated marginal means ± standard error (EMM ± SE) for BrdU+ cell count with subdivision volume or brain mass as a covariate. Values for aPOA, mPOA, and pPOA given in the top, middle, and bottom thirds respectively. Both covariates abolish the effect of status to some extent. Significant effects of status that survive correction for either covariate were followed by post-hoc analysis for pairwise differences, and superscript letters are used to indicate which groups did and did not differ from each other.

Status	BrdU+ Cell Counts (EMM ± SE) with Volume Covariate		with Brain Mass Covariate	
Anterior				
D	341.43	±26.59	318.95^{ab}	±30.10
S	280.20	±28.03	308.00^{ab}	±33.55
NF	524.52	±96.97	504.50^{a}	±101.15
NM	349.64	±96.01	387.98^{ab}	±101.12
F	256.35	±68.33	155.51^{b}	±83.90
M	376.92	±67.74	436.54^{a}	±70.94
Status	$F_{5,28} = 2.10, p = 0.096$		$\mathbf{F_{5,28} = 3.60, p = 0.01}$	
Volume	$\mathbf{F_{1,28} = 8.03, p = 0.008}$		–	
Brain Mass	–		$\mathbf{F_{1,28} = 7.77, p = 0.009}$	
Middle				
D	135.54^{ab}	±14.20	144.98^{ab}	±16.37
S	123.58^{ab}	±14.50	108.82^{a}	±17.83
NF	231.66^{a}	±52.16	256.22^{b}	±56.30
NM	113.29^{b}	±52.05	92.13^{a}	±56.29
F	55.23^{b}	±37.23	90.39^{a}	±44.87
M	114.93^{ab}	±36.74	103.65^{a}	±39.58
Status	$\mathbf{F_{5,28} = 3.29, p = 0.02}$		$\mathbf{F_{5,28} = 3.61, p = 0.012}$	
Volume	$\mathbf{F_{1,28} = 8.01, p = 0.009}$		–	
Brain Mass	–		$F_{1,28} = 0.59, p = 0.45$	
Posterior				
D	65.69	±5.26	64.93	±6.23
S	63.72	±5.46	63.48	±7.00
NF	99.33	±19.34	100.96	±20.64
NM	70.17	±19.30	69.95	±20.64
F	25.62	±14.66	33.66	±17.51
M	44.12	±13.65	43.43	±14.46
Status	$F_{5,28} = 2.29, p = 0.07$		$F_{5,28} = 1.90, p = 0.13$	
Volume	$\mathbf{F_{1,28} = 7.20, p = 0.01}$		–	
Brain Mass	–		$F_{1,28} = 8.03, p = 0.53$	

Table 2
Statistics and observed means ± standard error for POA subdivision volume and BrdU+ cell density. Values for aPOA, mPOA, and pPOA given in the top, middle, and bottom thirds respectively. Models for volume included brain mass as a covariate, while models for density did not. Status did not significantly affect volume or BrdU+ cell density in any subdivision.

Status	Volume (mm^3)		BrdU+ Cell Density	
Anterior				
D	0.0341	±0.0014	10611.42	±882.17
S	0.0280	±0.0011	9064.38	±602.86
NF	0.0388	±0.0034	15211.18	±4636.70
NM	0.0274	±0.0032	11723.45	±558.55
F	0.0361	±0.0046	8044.36	±1773.54
M	0.0343	±0.0034	11602.19	±4070.26
Status	$F_{5,28} = 1.06, p = 0.41$		$F_{5,29} = 1.75, p = 0.15$	
Brain Mass	$\mathbf{F_{1,28} = 17.64, p < 0.001}$		–	
Middle				
D	0.0224	±0.0011	6540.38	±888.65
S	0.0198	±0.0008	5790.75	±624.65
NF	0.0249	±0.0006	9981.62	±2139.71
NM	0.0186	±0.0042	5701.59	±1175.81
F	0.0255	±0.0023	2905.30	±830.61
M	0.0200	±0.0032	5404.60	±1146.25
Status	$F_{5,28} = 0.17, p = 0.97$		$F_{5,29} = 1.58, p = 0.20$	
Brain Mass	$\mathbf{F_{1,28} = 10.26, p = 0.003}$		–	
Posterior				
D	0.0143	±0.0007	4942.41	±422.53
S	0.0128	±0.0005	4802.59	±498.71
NF	0.0155	±0.0023	7014.94	±1974.25
NM	0.0127	±0.0024	5594.54	±1873.59
F	0.0186	±0.0022	2084.29	±441.21
M	0.0130	±0.0006	3303.44	±960.13
Status	$F_{5,28} = 0.82, p = 0.55$		$F_{5,29} = 1.55, p = 0.20$	
Brain Mass	$\mathbf{F_{1,28} = 20.10, p < 0.001}$		–	

in the mPOA was again driven by new females (see Table 1 for pairwise differences). There were no effects of status on BrdU+ cell density or on volume in any POA subregion (Table 2). Based on these results we cannot rule out the hypothesis that the differences in BrdU+ cell count observed here are at least partially attributable to differences in POA size. Still, it is noteworthy that an effect of status was only detected in the aPOA, but not the mPOA or pPOA, for BrdU+ cell count over time when omitting brain mass or POA volume as a covariate (Fig. 5A).

4. Discussion

The goal of this study was to test whether a period of enhanced cell addition to the aPOA could be identified that may contribute to the emergence of a sex difference in cell number during sex change, and whether that process relates to gonadal sex change. A sex difference was previously described in the anemonefish aPOA, such that females display approximately twice the number of medium-sized cells than males (Dodd et al., 2019). This sex difference is observable in sex-changing fish several months after the initiation of sex change, before gonadal sex change occurs and before the circulating sex hormones are female-like (Dodd et al., 2019). In the present study we found that sex-changing fish added more new cells to the aPOA, but not the mPOA or pPOA, than their non-changing partners (Fig. 5). Moreover, new female fish had even more new cells added to the aPOA than most (~75 % of) sex-changing fish, as well as to the mPOA. This may indicate that a further surge of new cell addition occurs around the time of gonadal sex change, but with only n = 2 new females confirmation in a larger sample will be necessary before generalizations can be made. Altogether, the results presented here are consistent with sex-changing fish gradually accumulating new cells in the aPOA throughout the months-long process of sex change. This effect is observable as an effect of status only in the aPOA where a sex difference was expected to gradually emerge, and not in the mPOA or pPOA where no sex difference was expected (Dodd et al., 2019). This accumulation occurs at a steady rate, meaning the effect can be masked when including stronger predictors of BrdU+ cell count like POA volume or brain size as a covariate. The increased cell addition throughout sex change may be a result of altered rates of cell proliferation, survival, migration, or differentiation, alone or in combination.

While new cells are being added to the aPOA of sex changing fish the proportion of testicular tissue decreases, but these two phenomena are probably unrelated because the same decrease in proportion of testicular tissue also occurs in the subordinate fish. Sex-changing fish and their non-changing partners (both of which were reproductive males before the experiment began) lost testicular tissue to a comparable degree within the first 6 weeks after initiation of sex change, and this reduced testicular proportion was maintained throughout the experiment. Because control males displayed a higher proportion of testicular tissue than dominant or subordinate fish, this indicates that the proportion of testicular tissue decreases substantially within the first 6 weeks in both dominant and subordinate fish. This reduced testicular proportion is continuously maintained for at least 26 weeks, and if anything slowly continues to decline as seen in previous work (Dodd et al., 2019). This is consistent with the notion that loss of testicular tissue may reflect a state of reproductive stasis that both sex-changing and non-changing fish occupy, and that the loss of testicular tissue in a sex-changing fish need not necessarily reflect a process of feminization *per se* (Dodd et al., 2019; Fricke, 1983).

Further, we find no clear relationship between gonadal hormones and the addition of new cells to the aPOA. This study was carried out in coordination with a separate study that quantified circulating androgen (11-ketotestosterone; 11KT) and estrogen (estradiol; E2) from plasma samples taken the day of tissue collection in these fish (Parker et al., 2022). Hormones aligned with gonadal histology (Fig. 3) such that dominant and subordinate fish had levels of 11KT and E2 in-between

what was observed in control males (high 11KT and low E2) or control females (high E2 and low 11KT), and hormones did not significantly change over time in either group. Dominant and subordinate fish had comparable levels of E2, but dominant fish had significantly higher levels of 11KT compared to subordinate fish (although still significantly lower than a control male). New females and new males had hormone profiles more similar to their new sex: 11KT in new females dropped to a control female level while E2 rose, and 11KT in new males rose to a control male level while E2 dropped. Elevated 11KT in sex-changing fish may reflect a role in driving cell proliferation, as recent work in tilapia has demonstrated a stimulatory role for 11KT in brain cell proliferation and neurogenesis (Narita et al., 2018). Alternatively, the increased addition of new cells to the aPOA in sex-changing fish could result from mechanisms that are intrinsic to the brain and independent of the gonads and gonadal hormones.

Mechanisms underlying sexual differentiation of the brain that are independent of gonads and gonadal hormones remain poorly understood. Alternative mechanisms that may be responsible for the observed effects include sex steroids synthesized locally within the brain, or glucocorticoid signaling. Estradiol synthesized within the brain by radial glia has an inhibitory effect on adult neurogenesis in fish, and the expression of brain aromatase (the enzyme that synthesizes estradiol) is dynamically regulated in order to actively modulate rates of neurogenesis in the context of brain repair after injury in zebrafish (Diotel et al., 2013; Pellegrini et al., 2016). However, brain aromatase expression *increases* in anemonefish throughout sex change (Casas et al., 2016), which would be inconsistent with increased new cell addition in the aPOA if E2 is inhibitory in these fish as well. These data on brain aromatase expression throughout sex change were collected from whole-brain homogenate, so it may be possible that aromatase is down-regulated locally within the aPOA to create a niche favorable to cell proliferation despite being upregulated at the whole-brain level. Glucocorticoids (e.g. cortisol) are another alternative mechanism, having been identified as mediating the effects of social stimuli on neurogenesis in a series of studies in weakly electric fish (Dunlap et al., 2013, 2011, 2006). In these fish, social enrichment is associated with elevated cortisol that in turn drives increased neurogenesis within brain regions responsible for social communication. Cortisol is elevated during sex change in anemonefish above what is seen in either males or females (Godwin and Thomas, 1993), and elevated glucocorticoid signaling is thought to play a central role in many models of socially-controlled sex change (Goikoetxea et al., 2017; Solomon-Lane et al., 2013; Todd et al., 2019). An analogous process of brain region-specific, cortisol-induced new cell addition may underlie sexual differentiation of the aPOA of anemonefish, but further work is needed. Identifying the signaling pathways that connect dominance status to increased cell addition to specific regions of the POA would greatly advance our understanding of how gonad-independent sexual differentiation of the brain is accomplished.

Our findings are consistent with past work indicating that the gonads of sex-changing fish and their non-changing partners lost testicular tissue in unison after pairing (Dodd et al., 2019). This has a number of implications for how protandrous sex change in anemonefish is unique relative to protogynous or bidirectional sex change. First, it supports the theory that gonadal sex change in anemonefish is not a linear progression directly from male to female. Rather, the gonads of sex-changing fish shift to a state of non-reproductive quiescence while body growth is prioritized (Buston, 2003; Fricke, 1983; Parker et al., 2022) and changes take place in the brain (Dodd et al., 2019; Elofsson et al., 1997). Some previous studies that examined sex change in wild anemonefish interpreted the predominance of non-vitellogenic ovarian tissue in the gonads of sex-changing fish as indicating partial gonadal sex change or an immature female phenotype (Casas et al., 2016; Godwin, 1994), including one of the earliest pioneering reports of experimentally-induced sex change in anemonefish by Fricke & Fricke (1977). However, in subsequent work Hans Fricke corrected this interpretation, preferring instead the theory that the gonads of these fish are arrested at a critical stage just before the irreversible commitment to a female fate, retaining the ability to reverse course if needed (Fricke, 1983). By sampling both sex-changing fish and their non-changing partners and revealing their gonadal composition to be comparable to one another in this study and past work (Dodd et al., 2019), we find further support for this reproductive quiescence model to explain the gonadal state of sex-changing fish. This state of reproductive quiescence in both sex-changing and non-changing fish may be similar to the gonadal state observed in non-breeding anemonefish. Non-breeding anemonefish also have gonads that are primarily composed of non-vitellogenic ovarian tissue (Wang et al., 2022) similar to what we have observed in sex-changing and non-changing fish here and in past work (Dodd et al., 2019). Yet the gonads of non-breeding anemonefish are transcriptomically more male-like than female-like (Wang et al., 2022), which underscores the notion that in anemonefish a gonad that is overwhelmingly composed of non-vitellogenic ovarian tissue is not necessarily female-like. And while other work has found the overall gonadal transcriptome of sex-changing anemonefish to be distinct from both males and females (Casas et al., 2016), it remains to be seen how the gonads of sex-changing fish compare to those of their non-changing partners at the molecular level.

Second, these results reinforce the hypothesis that the timecourse of gonadal sex change in anemonefish is protracted and highly variable, unlike in protogynous or bidirectional species. In this and past work from our lab, the earliest observed sex change was within four months, and by six months sex change was completed in approximately 25 % of pairs (Dodd et al., 2019; Parker et al., 2022). From other past work (much of which was done in the field with wild fish) sex change is typically completed anywhere between two to six months (Casas et al., 2016; Fricke and Fricke, 1977; Fricke, 1983; Godwin, 1994; Moyer and Nakazono, 1978), with the shortest reported timespan for complete sex change at 26 days (Fricke and Fricke, 1977). Contrast this with the protogynous Bluehead wrasse or the bidirectional sex-changing bluebanded goby, both of which predictably complete sex change within two to three weeks (Reavis and Grober, 1999; Todd et al., 2019; Warner and Swearer, 1991). It appears, then, that gonadal sex change in anemonefish is uniquely characterized by a slow and individually-variable progression, making it distinct from other modes of sex change as they are currently understood. The final commitment to a female gonadal fate is likely approached with caution because it is irreversible (Fricke and Fricke, 1977; Fricke, 1983), and the “decision” to commit is probably dependent on intrinsic factors related to body growth and preparedness to spawn (Buston, 2003; Fricke, 1983; Reed et al., 2019; Warner, 1988). It may be that (re)differentiation of the brain precedes, and possibly determines, differentiation of the gonads, as has been proposed for teleost fish generally (Francis, 1992). During this period, circulating 11KT and E2 remains between male- and female-typical levels until the final commitment to a female gonadal fate (Dodd et al., 2019; Godwin and Thomas, 1993; Parker et al., 2022) while the POA is gradually differentiating. Distinct timecourses for transformation of different tissue types suggest that sex change involves the coordination of multiple tissue-specific mechanisms for sexual differentiation (Arnold, 2020). Future work should continue to leverage sex-changing fishes for the study of sexual differentiation coordinated across the whole organism (for example, see Lorenzi et al., 2012; Schuppe et al., 2017). These data also highlight the potential for comparisons between lineages of sex-changing fish to reveal divergent, convergent, or conserved patterns and mechanisms of sexual plasticity, given that socially-controlled sex change has independently evolved in numerous teleost families (de Mitcheson and Liu, 2008; Erisman et al., 2013; Pla et al., 2022).

One limitation of this study is that we did not evaluate the phenotype of the BrdU+ cells, and so we do not know what proportion of the BrdU+ cells are neurons, radial glia, or other cell types. BrdU+ cells were observed throughout the POA, near the ventricle as well as deeper into the brain parenchyma. Cells deeper into the brain parenchyma are likely

to be neurons as few other cell types populate the POA in large number, and some portion of the cells nearer the ventricle may be radial glia serving as a progenitor cell pool (Cuoghi and Mola, 2009; Ganz and Brand, 2016). Which specific types of neurons (e.g. excitatory, inhibitory, neuropeptide-expressing, steroid sensitive) or glia (e.g. aromatase expressing, progenitor cell) populate the POA during sex change is an important question for future inquiry. Further research is also needed to test which component processes (differential cell proliferation, survival, migration or differentiation) contribute to growth of this cell population.

Likewise, the function of these aPOA cells is not yet known. They may serve to differentiate some aspect of physiology, as differentiated cell numbers in the mammalian anterior POA differentiates reproductive physiology (McCarthy, 2020; Mohr et al., 2017). Indeed, the present study parallels a series of studies in rats that used similar methods to establish that new cells (both neurons and glia) are gradually added to the AVPV during the peripubertal period (Ahmed et al., 2008; Mohr et al., 2016). Those new cells help regulate the luteinizing hormone surge that is necessary for ovulation in female rats (Mohr et al., 2017). The timecourse of POA sex change described here is consistent with a role in regulating differentiated physiology or behavior, as differentiation of the POA precedes both gonadal and behavioral sex change (Dodd et al., 2019; Parker et al., 2022). Alternatively, sex differences in the developing or adult brain can in some cases bring the sexes toward similar functional endpoints by compensating for differences elsewhere in the system (Arnold, 2014; De Vries, 2004; De Vries and Boyle, 1998; McCarthy et al., 2012). The classic example of this comes from research in biparental prairie voles (De Vries, 2004). Female and male prairie voles display a similar degree and kind of parental care behavior (with the obvious exception of nursing). In females this behavior is driven by parturition-associated hormonal changes. Males, however, do not give birth and so rely on a sexually-differentiated vasopressin system to drive parental care behavior. If future research determines that the new cells in the anemonefish POA are involved in regulating the female reproductive cycle or some aspect of sexually-differentiated social behavior, then anemonefish may provide a valuable model for investigating the conserved or divergent mechanisms for differentiation of the female reproductive system. Results from such research will, in turn, benefit our understanding of female brain differentiation and reproductive function across vertebrate species.

5. Conclusions

During male-to-female sex change in anemonefish, new cells are gradually added to the anterior POA before the gonads become female-like and before the fish displays female-typical sex hormones. These effects may be mediated by gonadal steroids, or by gonad-independent mechanisms like brain-derived steroids or systemic glucocorticoids that are secreted during sex change. These results provide further insight into the unique characteristics of protandrous sex change in anemonefish relative to other modes of sex change in other species. In doing so, they reinforce the usefulness of anemonefish as a complementary model to other sex-changing fish, and the potential for developing a richer understanding of the myriad mechanisms for achieving sexual differentiation of the brain.

CRediT authorship contribution statement

Coltan G. Parker: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Sarah E. Craig:** Investigation. **Abigail R. Histed:** Investigation. **Joanne S. Lee:** Investigation. **Emma Ibanez:** Investigation. **Veronica Pronitcheva:** Investigation. **Justin S. Rhodes:** Resources, Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by start-up funds and indirect costs recovered from federal grants to JSR.

References

Ahmed, E.I., Zehr, J.L., Schulz, K.M., Lorenz, B.H., DonCarlos, L.L., Sisk, C.L., 2008. Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nat. Neurosci. 11, 995–997. https://doi.org/10.1038/nn.2178.

Allen, L.S., Hines, M., Shryne, J.E., Gorski, R.A., 1989. Two sexually dimorphic cell groups in the human brain. J. Neurosci. 9, 497–506. https://doi.org/10.1523/JNEUROSCI.09-02-00497.1989.

Arnold, A.P., 2014. Conceptual frameworks and mouse models for studying sex differences in physiology and disease: Why compensation changes the game. Exp. Neurol. Special Issue: The importance of sex in the etiology, presentation, and treatment of neurological disorders 259, 2–9. https://doi.org/10.1016/j.expneurol.2014.01.021.

Arnold, A.P., 2020. Sexual differentiation of brain and other tissues: Five questions for the next 50 years. Horm. Behav. 120, 104691 https://doi.org/10.1016/j.yhbeh.2020.104691.

Balthazart, J., Ball, G.F., 2007. Topography in the preoptic region: Differential regulation of appetitive and consummatory male sexual behaviors. Front. Neuroendocrinol. 28, 161–178. https://doi.org/10.1016/j.yfrne.2007.05.003.

Balthazart, J., Cornil, C.A., Charlier, T.D., Taziaux, M., Ball, G.F., 2009. Estradiol, a key endocrine signal in the sexual differentiation and activation of reproductive behavior in quail. J. Exp. Zool. Part Ecol. Genet. Physiol. 311A, 323–345. https://doi.org/10.1002/jez.464.

Black, M.P., Reavis, R.H., Grober, M.S., 2004. Socially induced sex change regulates forebrain isotocin in Lythrypnus dalli. Neuroreport 15 (1), 185–189.

Bleier, R., Byne, W., Siggelkow, I., 1982. Cytoarchitectonic sexual dimorphisms of the medial preoptic and anterior hypothalamic areas in guinea pig, rat, hamster, and mouse. J. Comp. Neurol. 212, 118–130. https://doi.org/10.1002/cne.902120203.

Bloch, G.J., Gorski, R.A., 1988. Cytoarchitectonic analysis of the SDN-POA of the intact and gonadectomized rat. J. Comp. Neurol. 275, 604–612. https://doi.org/10.1002/cne.902750408.

Boyd, S.K., Tyler, C.J., de Vries, G.J., 1992. Sexual dimorphism in the vasotocin system of the bullfrog (Rana catesbeiana). J. Comp. Neurol. 325, 313–325. https://doi.org/10.1002/cne.903250213.

Burbridge, S., Stewart, I., Placzek, M., 2016. Development of the Neuroendocrine Hypothalamus, in: Comprehensive Physiology. John Wiley & Sons, Ltd, pp. 623–643 https://doi.org/10.1002/cphy.c150023.

Buston, P.M., 2003. Size and growth modification in clownfish. Nature 424, 145–146. https://doi.org/10.1038/424145a.

Byne, W., Lasco, M.S., Kemether, E., Shinwari, A., Edgar, M.A., Morgello, S., Jones, L.B., Tobet, S., 2000. The interstitial nuclei of the human anterior hypothalamus: an investigation of sexual variation in volume and cell size, number and density. Brain Res. 856, 254–258. https://doi.org/10.1016/S0006-8993(99)02458-0.

Casas, L., Parker, C.G., Rhodes, J.S., 2022. Sex change from male to female: Active feminization of the brian, behavior, and gonads in anemonefish. In: Evolution, Development, and Ecology of Anemonefishes: Model Organisms for Marine Science, pp. 117–128.

Casas, L., Saborido-Rey, F., 2021. Environmental Cues and Mechanisms Underpinning Sex Change in Fish. Sex. Dev. 15 (1-3), 108–121.

Casas, L., Saborido-Rey, F., Ryu, T., Michell, C., Ravasi, T., Irigoien, X., 2016. Sex Change in Clownfish: Molecular Insights from Transcriptome Analysis. Sci. Rep. 6, 35461. https://doi.org/10.1038/srep35461.

Clarkson, J., Herbison, A.E., 2006. Postnatal Development of Kisspeptin Neurons in Mouse Hypothalamus; Sexual Dimorphism and Projections to Gonadotropin-Releasing Hormone Neurons. Endocrinology 147, 5817–5825. https://doi.org/10.1210/en.2006-0787.

Crews, D., Wade, J., Wilczynski, W., 1990. Sexually Dimorphic Areas in the Brain of Whiptail Lizards. Brain Behav. Evol. 36, 262–270. https://doi.org/10.1159/000115312.

Cuoghi, B., Mola, L., 2009. Macroglial cells of the teleost central nervous system: a survey of the main types. Cell Tissue Res. 338, 319–332. https://doi.org/10.1007/s00441-009-0870-2.

Davis, E.C., Shryne, J.E., Gorski, R.A., 1996. Structural Sexual Dimorphisms in the Anteroventral Periventricular Nucleus of the Rat Hypothalamus Are Sensitive to Gonadal Steroids Perinatally, but Develop Peripubertally. Neuroendocrinology 63, 142–148. https://doi.org/10.1159/000126950.

De Mitcheson, Y.S., Liu, M., 2008. Functional hermaphroditism in teleosts. Fish Fish. 9, 1–43. https://doi.org/10.1111/j.1467-2979.2007.00266.x.
De Vries, G.J., 2004. Minireview: Sex Differences in Adult and Developing Brains: Compensation, Compensation. Compensat. Endocrinol. 145, 1063–1068. https://doi.org/10.1210/en.2003-1504.
De Vries, G.J., Boyle, P.A., 1998. Double duty for sex differences in the brain. Behav. Brain Res. 92, 205–213. https://doi.org/10.1016/S0166-4328(97)00192-7.
DeAngelis, R., Dodd, L., Rhodes, J., 2020. Nonapeptides mediate trade-offs in parental care strategy. Horm. Behav. 121, 104717 https://doi.org/10.1016/j.yhbeh.2020.104717.
Diotel, N., Vaillant, C., Gabbero, C., Mironov, S., Fostier, A., Gueguen, M.-M., Anglade, I., Kah, O., Pellegrini, E., 2013. Effects of estradiol in adult neurogenesis and brain repair in zebrafish. Horm. Behav Hormones & Neurotrauma: Protection, Degeneration and Plasticity 63, 193–207. https://doi.org/10.1016/j.yhbeh.2012.04.003.
Dodd, L.D., Nowak, E., Lange, D., Parker, C.G., DeAngelis, R., Gonzalez, J.A., Rhodes, J. S., 2019. Active feminization of the preoptic area occurs independently of the gonads in Amphiprion ocellaris. Horm. Behav. 112, 65–76. https://doi.org/10.1016/j.yhbeh.2019.04.002.
Dunlap, K.D., Castellano, J.F., Prendaj, E., 2006. Social interaction and cortisol treatment increase cell addition and radial glia fiber density in the diencephalic periventricular zone of adult electric fish, Apteronotus leptorhynchus. Horm. Behav. 50, 10–17. https://doi.org/10.1016/j.yhbeh.2006.01.003.
Dunlap, K.D., Jashari, D., Pappas, K.M., 2011. Glucocorticoid receptor blockade inhibits brain cell addition and aggressive signaling in electric fish, Apteronotus leptorhynchus. Horm. Behav. 60, 275–283. https://doi.org/10.1016/j.yhbeh.2011.06.001.
Dunlap, K.D., Chung, M., Castellano, J.F., Krahe, R., Fortune, E., 2013. Influence of long-term social interaction on chirping behavior, steroid levels and neurogenesis in weakly electric fish. J. Exp. Biol. 216 (13), 2434–2441.
Elofsson, U., Winberg, S., Francis, R.C., 1997. Number of preoptic GnRH-immunoreactive cells correlates with sexual phase in a protandrously hermaphroditic fish, the dusky anemonefish (Amphiprionmelanopus). J. Comp. Physiol. A 181, 484–492. https://doi.org/10.1007/s003590050132.
Erisman, B.E., Petersen, C.W., Hastings, P.A., Warner, R.R., 2013. Phylogenetic Perspectives on the Evolution of Functional Hermaphroditism in Teleost Fishes. Integr. Comp. Biol. 53, 736–754. https://doi.org/10.1093/icb/ict077.
Fernald, R.D., Shelton, L.C., 1985. The organization of the diencephalon and the pretectum in the cichlid fish, Haplochromis burtoni. J. Comp. Neurol. 238, 202–217. https://doi.org/10.1002/cne.902380207.
Francis, R.C., 1992. Sexual Lability in Teleosts: Developmental Factors. Q. Rev. Biol. 67, 1–18. https://doi.org/10.1086/417445.
Fricke, H.W., 1979. Mating System, Resource Defence and Sex Change in the Anemonefish Amphiprion akallopisos. Z. Für Tierpsychol. 50, 313–326. https://doi.org/10.1111/j.1439-0310.1979.tb01034.x.
Fricke, H.W., 1983. Social Control of Sex: Field Experiments with the Anemonefish Amphiprion bicinctus. Z. Für Tierpsychol. 61, 71–77. https://doi.org/10.1111/j.1439-0310.1983.tb01327.x.
Fricke, H., Fricke, S., 1977. Monogamy and sex change by aggressive dominance in coral reef fish. Nature 266, 830–832. https://doi.org/10.1038/266830a0.
Ganz, J., Brand, M., 2016. Adult Neurogenesis in Fish. Cold Spring Harb. Perspect. Biol. 8 (7) https://doi.org/10.1101/cshperspect.a019018.
Garcia-Falgueras, A., Swaab, D.F., 2008. A sex difference in the hypothalamic uncinate nucleus: relationship to gender identity. Brain 131, 3132–3146. https://doi.org/10.1093/brain/awn276.
Godwin, J.R., 1994. Histological aspects of protandrous sex change in the anemonefish Amphiprion melanopus (Pomacentridae, Teleostei). J. Zool. 232, 199–213. https://doi.org/10.1111/j.1469-7998.1994.tb01569.x.
Godwin, J.R., Thomas, P., 1993. Sex Change and Steroid Profiles in the Protandrous Anemonefish Amphiprion melanopus (Pomacentridae, Teleostei). Gen. Comp. Endocrinol. 91, 144–157. https://doi.org/10.1006/gcen.1993.1114.
Goikoetxea, A., Todd, E.V., Gemmell, N.J., 2017. Stress and sex: does cortisol mediate sex change in fish? R149–R160 Reproduction 154. https://doi.org/10.1530/REP-17-0408.
Gonzalez, J.A., Histed, A.R., Nowak, E., Lange, D., Craig, S.E., Parker, C.G., Kaur, A., Bhuvanagiri, S., Kroll, K.J., Martyniuk, C.J., Denslow, N.D., Rosenfeld, C.S., Rhodes, J.S., 2021. Impact of bisphenol-A and synthetic estradiol on brain, behavior, gonads and sex hormones in a sexually labile coral reef fish. Horm. Behav. 136, 105043 https://doi.org/10.1016/j.yhbeh.2021.105043.
Gorski, R.A., Gordon, J.H., Shryne, J.E., Southam, A.M., 1978. Evidence for a morphological sex difference within the medial preoptic area of the rat brain. Brain Res. 148, 333–346. https://doi.org/10.1016/0006-8993(78)90723-0.
Grober, M.S., Bass, A.H., 1991. Neuronal Correlates of Sex/Role Change in Labrid Fishes: LHRH-Like Immunoreactivity. Brain Behav. Evol. 38, 302–312. https://doi.org/10.1159/000114396.
Grober, M.S., Sunobe, T., 1996. Serial adult sex change involves rapid and reversible changes in forebrain neurochemistry. Neuroreport 7 (18), 2945–2950.
Grober, M.S., Jackson, I.M.D., Bass, A.H., 1991. Gonadal steroids affect LHRH preoptic cell number in a sex/role changing fish. J. Neurobiol. 22, 734–741. https://doi.org/10.1002/neu.480220708.
Hillsman, K.D., Sanderson, N.S., Crews, D., 2007. Testosterone Stimulates Mounting Behavior and Arginine Vasotocin Expression in the Brain of both Sexual and Unisexual Whiptail Lizards. Sex. Dev. 1, 77–84. https://doi.org/10.1159/000096241.
Iwata, E., Manbo, J., 2013. Territorial behaviour reflects sexual status in groups of false clown anemonefish (Amphiprion ocellaris) under laboratory conditions. Acta Ethologica 16, 97–103. https://doi.org/10.1007/s10211-012-0142-0.
Juraska, J.M., Sisk, C.L., DonCarlos, L.L., 2013. Sexual differentiation of the adolescent rodent brain: Hormonal influences and developmental mechanisms. Horm. Behav. Puberty Adolescence 64, 203–210. https://doi.org/10.1016/j.yhbeh.2013.05.010.
Kanda, S., 2019. Evolution of the regulatory mechanisms for the hypothalamic-pituitary-gonadal axis in vertebrates–hypothesis from a comparative view. Gen. Comp. Endocrinol. 284, 113075 https://doi.org/10.1016/j.ygcen.2018.11.014.
Kaslin, J., Ganz, J., Brand, M., 2008. Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain. Philos. Trans. R. Soc. B Biol. Sci. 363, 101–122. https://doi.org/10.1098/rstb.2006.2015.
Kuwamura, T., Sunobe, T., Sakai, Y., Kadota, T., Sawada, K., 2020. Hermaphroditism in fishes: an annotated list of species, phylogeny, and mating system. Ichthyol. Res. 67, 341–360. https://doi.org/10.1007/s10228-020-00754-6.
Lamm, M.S., Liu, H., Gemmell, N.J., Godwin, J.R., 2015. The Need for Speed: Neuroendocrine Regulation of Socially-controlled Sex Change. Integr. Comp. Biol. 55, 307–322. https://doi.org/10.1093/icb/icv041.
LeVay, S., 1991. A difference in hypothalamic structure between heterosexual and homosexual men. Science 253, 1034–1037. https://doi.org/10.1126/science.1887219.
Lorenzi, V., Earley, R.L., Grober, M.S., Bartolomucci, A., 2012. Differential Responses of Brain, Gonad and Muscle Steroid Levels to Changes in Social Status and Sex in a Sequential and Bidirectional Hermaphroditic Fish. PLoS One 7 (12). https://doi.org/10.1371/journal.pone.0051158.
Madeira, M.D., Leal, S., Paula-Barbosa, M.M., 1999. Stereological evaluation and Golgi study of the sexual dimorphisms in the volume, cell numbers, and cell size in the medial preoptic nucleus of the rat. J. Neurocytol. 28, 131–148. https://doi.org/10.1023/A:1007076206828.
Maruska, K.P., Carpenter, R.E., Fernald, R.D., 2012. Characterization of cell proliferation throughout the brain of the African cichlid fish Astatotilapia burtoni and its regulation by social status. J. Comp. Neurol. 520, 3471–3491. https://doi.org/10.1002/cne.23100.
McCarthy, M.M., 2008. Estradiol and the Developing Brain. Physiol. Rev. 88, 91–134. https://doi.org/10.1152/physrev.00010.2007.
McCarthy, M.M., 2020. Origins of Sex Differentiation of Brain and Behavior. In: Wray, S., Blackshaw, S. (Eds.), Developmental Neuroendocrinology, Masterclass in Neuroendocrinology. Springer International Publishing, Cham, pp. 393–412. https://doi.org/10.1007/978-3-030-40002-6_15.
McCarthy, M.M., Arnold, A.P., Ball, G.F., Blaustein, J.D., Vries, G.J.D., 2012. Sex Differences in the Brain: The Not So Inconvenient Truth. J. Neurosci. 32, 2241–2247. https://doi.org/10.1523/JNEUROSCI.5372-11.2012.
Mohr, M.A., Garcia, F.L., DonCarlos, L.L., Sisk, C.L., 2016. Neurons and Glial Cells Are Added to the Female Rat Anteroventral Periventricular Nucleus During Puberty. Endocrinology 157, 2393–2402. https://doi.org/10.1210/en.2015-2012.
Mohr, M.A., DonCarlos, L.L., Sisk, C.L., 2017. Inhibiting Production of New Brain Cells during Puberty or Adulthood Blunts the Hormonally Induced Surge of Luteinizing Hormone in Female Rats. eNeuro 4 (5).
Moore, F.L., Richardson, C., Lowry, C.A., 2000. Sexual Dimorphism in Numbers of Vasotocin-Immunoreactive Neurons in Brain Areas Associated with Reproductive Behaviors in the Roughskin Newt. Gen. Comp. Endocrinol. 117, 281–298. https://doi.org/10.1006/gcen.1999.7424.
Moyer, J.T., Nakazono, A., 1978. Protandrous hermaphroditism in six species of the anemonefish genus Amphiprion in Japan. Jpn. J. Ichthyol. 25, 101–106.
Nakamura, M., Hourigan, T.F., Yamauchi, K., Nagahama, Y., Grau, E.G., 1989. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse Thalassoma duperrey. Environ. Biol. Fishes 24, 117–136. https://doi.org/10.1007/BF00001282.
Narita, Y., Tsutiya, A., Nakano, Y., Ashitomi, M., Sato, K., Hosono, K., Kaneko, T., Chen, R.-D., Lee, J.-R., Tseng, Y.-C., Hwang, P.-P., Ohtani-Kaneko, R., 2018. Androgen induced cellular proliferation, neurogenesis, and generation of GnRH3 neurons in the brain of mature female Mozambique tilapia. Sci. Rep. 8, 16855. https://doi.org/10.1038/s41598-018-35303-9.
O'Connell, L.A., Hofmann, H.A., 2011. The Vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. J Comp Neurol 519, 3599–3639. https://doi.org/10.1002/cne.22735.
O'Connell, L.A., Hofmann, H.A., 2012. Evolution of a Vertebrate Social Decision-Making Network. Science 336, 1154–1157. https://doi.org/10.1126/science.1218889.
Okubo, K., Miyazoe, D., Nishiike, Y., 2019. A conceptual framework for understanding sexual differentiation of the teleost brain. Gen. Comp. Endocrinol. 284, 113129 https://doi.org/10.1016/j.ygcen.2019.02.020.
Page, Y.L., Diotel, N., Vaillant, C., Pellegrini, E., Anglade, I., Mérot, Y., Kah, O., 2010. Aromatase, brain sexualization and plasticity: the fish paradigm. Eur. J. Neurosci. 32, 2105–2115. https://doi.org/10.1111/j.1460-9568.2010.07519.x.
Panzica, G., Viglietti-Panzica, C., Sanchez, F., Sante, P., Balthazart, J., 1991. Effects of testosterone on a selected neuronal population within the preoptic sexually dimorphic nucleus of the Japanese quail. J. Comp. Neurol. 303, 443–456. https://doi.org/10.1002/cne.903030310.
Parker, C.G., Lee, J.S., Histed, A.R., Craig, S.E., Rhodes, J.S., 2022. Stable and persistent male-like behavior during male-to-female sex change in the common clownfish Amphiprion ocellaris. Horm. Behav. 145, 105239 https://doi.org/10.1016/j.yhbeh.2022.105239.
Pellegrini, E., Diotel, N., Vaillant-Capitaine, C., Pérez Maria, R., Gueguen, M.-M., Nasri, A., Cano Nicolau, J., Kah, O., 2016. Steroid modulation of neurogenesis: Focus on radial glial cells in zebrafish. J. Steroid Biochem. Mol. Biol., SI: Steroids & Nervous System 160, 27–36. https://doi.org/10.1016/j.jsbmb.2015.06.011.

Pla, S., Benvenuto, C., Capellini, I., Piferrer, F., 2022. Switches, stability and reversals in the evolutionary history of sexual systems in fish. Nat. Commun. 13, 3029. https://doi.org/10.1038/s41467-022-30419-z.

Prim, J.H., Phillips, M.C., Lamm, M.S., Brady, J., Cabral, I., Durden, S., Dustin, E., Hazellief, A., Klapheke, B., Lamb, A.D., Lukowsky, A., May, D., Sanchez, S.G., Thompson, K.C., Tyler, W.A., Godwin, J., 2022. Estrogenic signaling and sociosexual behavior in wild sex-changing bluehead wrasses, Thalassoma bifasciatum. J. Exp. Zool. Part Ecol. Integr. Physiol. 337, 24–34. https://doi.org/10.1002/jez.2558.

Reavis, R.H., Grober, M.S., 1999. An integrative approach to sex change: social, behavioural and neurochemical changes in Lythrypnus dalli (Pisces). Acta Ethologica 2, 51–60. https://doi.org/10.1007/PL00012232.

Reed, C., Branconi, R., Majoris, J., Johnson, C., Buston, P., 2019. Competitive growth in a social fish. Biol. Lett. 15, 20180737. https://doi.org/10.1098/rsbl.2018.0737.

Roselli, C.E., Larkin, K., Resko, J.A., Stellflug, J.N., Stormshak, F., 2004. The Volume of a Sexually Dimorphic Nucleus in the Ovine Medial Preoptic Area/Anterior Hypothalamus Varies with Sexual Partner Preference. Endocrinology 145, 478–483. https://doi.org/10.1210/en.2003-1098.

Schuppe, E.R., Pradhan, D.S., Thonkulpitak, K., Drilling, C., Black, M., Grober, M.S., Rosenfeld, C.S., 2017. Sex differences in neuromuscular androgen receptor expression and sociosexual behavior in a sex changing fish. PLoS One 12 (5). https://doi.org/10.1371/journal.pone.0177711 e0177711–e0177711.

Semsar, K., Godwin, J., 2003. Social Influences on the Arginine Vasotocin System Are Independent of Gonads in a Sex-Changing Fish. J. Neurosci. 23, 4386–4393. https://doi.org/10.1523/JNEUROSCI.23-10-04386.2003.

Semsar, K., Godwin, J., 2004. Multiple mechanisms of phenotype development in the bluehead wrasse. Horm. Behav. 45, 345–353. https://doi.org/10.1016/j.yhbeh.2004.01.003.

Shevchouk, O.T., Ball, G.F., Cornil, C.A., Balthazart, J., 2019. Rapid testosterone-induced growth of the medial preoptic nucleus in male canaries. Physiol. Behav. 204, 20–26. https://doi.org/10.1016/j.physbeh.2019.02.007.

Sickel, McCarthy, 2000. Calbindin-D28k Immunoreactivity is a Marker for a Subdivision of the Sexually Dimorphic Nucleus of the Preoptic Area of the Rat: Developmental Profile and Gonadal Steroid Modulation. J. Neuroendocrinol. 12 (5), 397–402.

Solomon-Lane, T., Crespi, E., Grober, M., 2013. Stress and serial adult metamorphosis: multiple roles for the stress axis in socially regulated sex change. Front. Neurosci. 7, 210. https://doi.org/10.3389/fnins.2013.00210.

Swaab, D.F., Fliers, E., 1985. A sexually dimorphic nucleus in the human brain. Science 228, 1112–1115. https://doi.org/10.1126/science.3992248.

Takami, S., Urano, A., 1984. The volume of the toad medial amygdala-anterior preoptic complex is sexually dimorphic and seasonally variable. Neurosci. Lett. 44, 253–258. https://doi.org/10.1016/0304-3940(84)90031-4.

Todd, E.V., Ortega-Recalde, O., Liu, H., Lamm, M.S., Rutherford, K.M., Cross, H., Black, M.A., Kardailsky, O., Graves, J.A.M., Hore, T.A., Godwin, J.R., Gemmell, N.J., 2019. Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change. Sci. Adv. 5, eaaw7006. https://doi.org/10.1126/sciadv.aaw7006.

Viglietti-Panzica, C., Panzica, G.C., Fiori, M.G., Calcagni, M., Anselmetti, G.C., Balthazart, J., 1986. A sexually dimorphic nucleus in the quail preoptic area. Neurosci. Lett. 64, 129–134. https://doi.org/10.1016/0304-3940(86)90087-X.

Wang, H., Qu, M., Tang, W., Liu, S., Ding, S., 2022. Transcriptome Profiling and Expression Localization of Key Sex-Related Genes in a Socially-Controlled Hermaphroditic Clownfish. Amphiprion clarkii. Int. J. Mol. Sci. 23, 9085. https://doi.org/10.3390/ijms23169085.

Warner, R.R., 1988. Sex change and the size-advantage model. Trends Ecol. Evol. 3, 133–136. https://doi.org/10.1016/0169-5347(88)90176-0.

Warner, R.R., Swearer, S.E., 1991. Social Control of Sex Change in the Bluehead Wrasse, Thalassoma bifasciatum (Pisces: Labridae). Biol. Bull. 181, 199–204. https://doi.org/10.2307/1542090.

Wong, M.Y.L., Medina, A., Uppaluri, C., Arnold, S., Seymour, J.R., Buston, P.M., 2013. Consistent behavioural traits and behavioural syndromes in pairs of the false clown anemonefish Amphiprion ocellaris. J. Fish Biol. 83, 207–213. https://doi.org/10.1111/jfb.12133.

Yaeger, C., Ros, A.M., Cross, V., DeAngelis, R.S., Stobaugh, D.J., Rhodes, J.S., 2014. Blockade of arginine vasotocin signaling reduces aggressive behavior and c-Fos expression in the preoptic area and periventricular nucleus of the posterior tuberculum in male Amphiprion ocellaris. Neuroscience 267, 205–218. https://doi.org/10.1016/j.neuroscience.2014.02.045.

Yamashita, J., Nishiike, Y., Fleming, T., Kayo, D., Okubo, K., 2021. Estrogen mediates sex differences in preoptic neuropeptide and pituitary hormone production in medaka. Commun. Biol. 4, 1–15. https://doi.org/10.1038/s42003-021-02476-5.

Zupanc, G.K.H., 2021. Adult neurogenesis in the central nervous system of teleost fish: from stem cells to function and evolution. J. Exp. Biol. 224, jeb226357. https://doi.org/10.1242/jeb.226357.