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# 12 Sex Change from Male to Female *Active Feminization of the Brain, Behavior, and Gonads in Anemonefish*

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## CONTENTS

12.1 Introduction .....	117
12.1.1 Who, When, and Why?.....	117
12.1.2 The Particular Case of Anemonefishes .....	118
12.2 Active Feminization of the Brain and Behavior .....	118
12.2.1 Initiation of Sex Change .....	120
12.2.2 POA Sex Change .....	120
12.2.3 Behavioral Sex Change.....	120
12.2.3.1 Sex Change in Aggression .....	120
12.2.3.2 Sex Change in Parental Care .....	121
12.2.3.3 Neural Mechanisms of Behavioral Sex Change .....	122
12.3 Gonadal Sex Change .....	122
12.3.1 Histological Changes across Sex Change in <i>Amphiprion</i> .....	122
12.3.2 Endocrine Regulation of Sex Change: Sex Steroids.....	123
12.3.3 Molecular Pathways Underlying Gonadal Sex Change.....	124
12.4 Conclusion .....	125
References.....	125

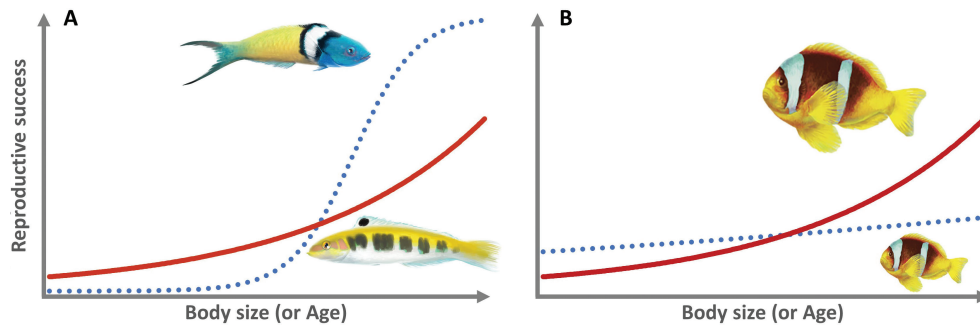
## 12.1 INTRODUCTION

In the majority of vertebrates, including fishes, sex is determined during early development and remains unchanged throughout life. Nonetheless, a small number of teleosts have the capacity to change functional sex in adulthood, exhibiting environmental sex determination. This unique sexual strategy is called sequential hermaphroditism and occurs sporadically among fish. It has been described in 462 species from 41 families and 17 orders, accounting for about 1.5% of teleosts (Nelson et al. 2016; Kuwamura et al. 2020). The majority of these species occur in the marine realm, especially on coral reefs, as only 2% of fish with the capacity to change sex inhabit freshwaters (Pandian 2010; Kuwamura et al. 2020). Three different forms of sex change have been described among sequentially hermaphroditic fishes: protandrous (fish mature first as males and then change to females), protogynous (fish mature first as females and then change to males), and bidirectional (fish can change back and forth between male and female phases). The most common type of sequential hermaphroditism in teleosts is protogyny (female first) with a prevalence five times higher than the two other strategies (Kuwamura et al. 2020).

### 12.1.1 WHO, WHEN, AND WHY?

The size-advantage model (SAM) is the most accepted theory to explain the occurrence of sex change and predict the timing and direction of the switch (Ghiselin 1969; Warner 1975, 1988; Munday et al. 2006). According to SAM, sex change should be favoured when the reproductive success of the opposite sex is enhanced at larger body sizes than the initial sex (Figure 12.1). Thus, the time at which sex change occurs is determined to maximize the lifetime reproductive output of an individual.

Factors triggering sex change in teleosts differ among species but are generally either size (and/or age) dependent or socially regulated and are closely related to the mating system and social structure of the species (Munday et al. 2006; Godwin 2009; Kobayashi et al. 2013). In most protogynous species, sex change is mediated by variations in the social context as they display mainly polygynous mating systems (Warner 1984; Kuwamura and Nakashima 1998; Munday et al. 2006). Most protandrous species need to attain a threshold age or size to change sex. One interesting exception are the anemonefishes (subfamily Amphiprioninae) since sex change is regulated socially in this group despite being protandrous hermaphrodites.



**FIGURE 12.1** Graphical representation of the size-advantage model of sex change. A) Protogyny is characterized by a greater increase of reproductive success with age/size in males than females. The bluehead wrasse is among the best-studied female-to-male sex changers and has proven to be a useful model for understanding protogyny. In B) protandry, reproductive success increases faster with age/size in females than in males. Anemonefishes are iconic representatives of protandrous sex change and have been used as models to investigate male-to-female sex change. Red (solid line) and blue (dotted line) indicate female and male growth curves, respectively. Modified from Warner (1975).

### 12.1.2 THE PARTICULAR CASE OF ANEMONEFISHES

Anemonefishes display a distinctive breeding strategy among teleosts. All studied species are monogamous protandrous hermaphrodites with strictly structured societies where sex change is controlled socially (Fricke and Fricke 1977; Moyer and Nakazono 1978; Ross 1978a; Fricke 1983; Godwin and Thomas 1993; Godwin 1994b). Anemonefishes live in pairs or groups organized by a strong size hierarchy that functions as a breeding queue. Within each group, the largest individual is always the female, surrounded by a male and a variable number of immature juvenile non-breeders. If the dominant female disappears, all her subordinates take the opportunity to ascend in rank and grow. The mature male that assumes the dominant position changes sex, while the largest immature fish differentiates into a mature male to complete the breeding pair. It is believed that ecological and social constraints lie at the heart of this particular breeding strategy (Rueger et al. 2021). Anemonefishes live in close association with cnidarian hosts that provide shelter from predators, food, and a refuge to lay their eggs (Fautin 1986, 1992). The patchy distribution of anemone hosts makes travel across the reef dangerous for a widowed fish in search of a new mate. Sex change allows anemonefishes to circumvent this danger (Elliott et al. 1995).

Similarly, non-breeders choose to forgo reproduction and avoid the risk of moving between hosts since inheritance of the territory provides the potential for future reproduction (Buston 2004; Rueger et al. 2021). Field observations in reefs of Japan have reported the presence of an individual of *A. clarkii* in the same anemone for 14+ years, going from subordinate immature to dominant male and subsequently changing sex to female (Moyer, 1986). The social hierarchy regulates the growth of subordinates to maintain defined size differences between individuals adjacent in rank, diminishing the risk of eviction due to direct competition for breeding positions (Buston 2003; Buston and Cant 2006; Branconi et al. 2020).

This breeding strategy is costly, not only energetically but also in terms of time. Thus, in more favourable

environments where the danger of movement between host anemones is reduced, social assemblages and pair bonds are not as stable, at least in some anemonefish species. In environments with a high density of host anemones, migration of mature males between territories to establish new breeding pairs is more frequent as it saves the time and energy required to change sex, a period during which the fish are not able to reproduce (Ochi 1989a). Moreover, when a breeding space is vacated, large non-breeders in the vicinity, migrate to fulfil the vacated position (Ochi 1989b). Movement of non-breeders among social groups is promoted as it favours rank improvement faster than waiting for a vacancy in its own group.

Sex change involves coordinated changes along multiple body axes. Once the dominance hierarchy is altered, rapid neurochemical changes in the brain reflect the upgraded status of social sex changers by adjusting the behavior within minutes to hours. The male, previously dominated by the female, starts displaying aggression and dominance. Subsequently, changes in the brain are transmitted to the gonads triggering a complete reorganization of the gonadal tissue. Together, a coordinated cascade of behavioral, physiological, and morphological changes results in a functional male becoming a functional female.

## 12.2 ACTIVE FEMINIZATION OF THE BRAIN AND BEHAVIOR

The great majority of what is known about sexual differentiation of the brain and periphery comes from mammalian models, where feminization occurs “by default”. Whereas masculinization is driven by gonadal hormones which shift the trajectory of development from female to male (McCarthy 2008; McCarthy et al. 2017; Tsukahara and Morishita 2020), feminization occurs in absence of gonadal steroids. However, just because a process occurs in the absence of gonadal hormones, does not mean it is a passive process. Evidence in mice establishes active proliferation and survival of cells in the brain and active suppression

of masculinizing genes (Ahmed et al. 2008; Nugent et al. 2015; Mohr et al. 2016). Hence, at least in mammals, feminization is not a “passive” process so much as it is a developmental process without a specific identifiable trigger like gonadal hormones, and thus the mechanisms have been more difficult to identify and sort out. The anemonefish represent an outstanding model to study the process of active feminization. Feminization is “active” in anemonefishes in the sense that female sexual differentiation follows from a social trigger and involves complete transformation from male to female, and so the active mechanisms can be tracked in real time as the fish transforms. This is less tractable in a mammalian model or other vertebrate models that display genetic sex determination or sex change from female to male in which there is no clear signal initiating the cascade of feminization.

There are three conceptually distinct stages of the brain’s involvement in the process of sex change in anemonefish.

1) *Initiation of sex change*: the brain senses and perceives ascent to dominant status and initiates sex change. 2)

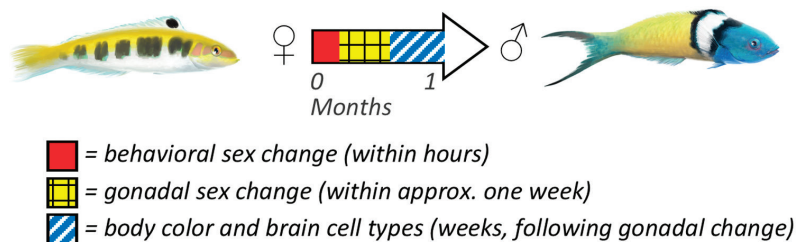
*Behavioral sex change*: male fish behave differently from female fish, and these behavioral differences must result from changes in physiology, connectivity, and/or activation of certain brain circuits. 3) *Hypothalamic sex change*: major changes in the numbers and types of neurons and glia in the preoptic area (POA) of the hypothalamus (and likely other hypothalamic sources of gonadotroph innervation, including the ventral tuberal and mediobasal hypothalamus) are needed to appropriately regulate the male versus female gonad via the hypothalamic-pituitary-gonad axis (Elofsson et al. 1997; Zohar et al. 2010; Trudeau 2018; Dodd et al. 2019).

The time course and process that ensues for producing female behavior and a female POA have not been fully worked out for anemonefishes (Dodd et al. 2019). Nevertheless, the collective evidence suggests the process is likely very different from protogynous (female-to-male) sex change (Figure 12.2).

The beginning is the same: the fish perceive a change in the social hierarchy. However, in protogynous sex change,

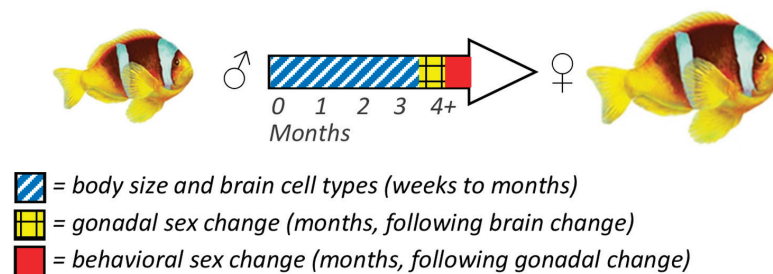
### A Protogynous sex change in the Bluehead Wrasse

Fast, gonad changes before morphology



### B Protandrous sex change in the Anemonefish

Slow, morphology changes before gonad



**FIGURE 12.2** Time-course of sex change from female to male in the bluehead wrasse as compared to male to female sex change in anemonefish. A) Female-to-male sex change in the bluehead wrasse is well established to occur in less than one month (Warner and Swearer 1991). Behavioral sex change occurs first, before gonadal and hormonal sex change. Masculinization of the gonads, which occurs within a week, precedes and orchestrates morphological changes in the POA and periphery via male sex hormones (Grober et al. 1991). B) In contrast, male-to-female sex change in anemonefish is less well understood. The process occurs over a wider time frame. In some species, gonadal sex change can be completed relatively rapidly after female removal (e.g., 26 days in *A. bicinctus* or 45 days in *A. melanopus* [Fricke and Fricke 1977; Godwin 1994a] while for *A. ocellaris* and other species [Moyer and Nakazono 1978; Hattori 1991; Godwin 1994a; Dodd et al. 2019] it takes four months or longer). In anemonefishes, while aggressive and dominance-associated behavior changes nearly immediately in response to ascension in the dominance hierarchy, the full behavioral phenotype is not intermediate between male and female and is more male-like than female-like. Behavioral sex change in anemonefishes occurs after gonadal sex change and after the sex steroids have completely changed. Hence, the process is nearly opposite to that described for the bluehead wrasse and other protogynous species.

behavior changes to that of the opposite sex within hours to days, well before the gonads change. Within a few days to weeks the brain signals to the gonads to change, and testicular tissue develops (Warner and Swearer 1991; Todd et al. 2019). Then, androgens are released from the gonads to complete the morphological sex change, including alterations in body size, changes in colouration and changes in numbers of GnRH neurons in the anterior POA that are involved in regulating gonadal function (Grober et al. 1991; Warner and Swearer 1991; Semsar and Godwin 2004; Todd et al. 2019). In anemonefishes, gonadal sex change (as defined by both the presence of vitellogenic oocytes in the gonad and the presence of a female-typical sex hormone profile) appears to be highly variable and can take a long time, up to years (described in more detail in the following section, “Gonadal Sex Change”). However, changes in the numbers of cells in the anterior POA appear to follow a predictable time-course and are completed in several months (Dodd et al. 2019). The precise time-course of cell addition to the POA has yet to be evaluated. The gonadal hormones (androgen and estrogen) of sex-changing fish are also in between male and female levels, but are overall more male-like than female-like (Dodd et al. 2019). The implication is that in anemonefishes, the parts of the brain that control the gonads (e.g., POA) change sex first, while the gonads and behavior revert to a reproductively dormant state not unlike that seen in juvenile non-breeder fish. When the fish is ready (and it is not clear exactly what constitutes “readiness”, perhaps a certain threshold of body growth or brain change), these brain parts signal to the gonads to feminize, and the sex hormone profile follows after vitellogenic eggs develop (Dodd et al. 2019).

### 12.2.1 INITIATION OF SEX CHANGE

The sensory stimuli that inform the male of his new dominant status remain to be determined. These stimuli likely include visual interpretation of behavioral displays and interactions with the other fish in the group, possible detection of pheromones, changes in the perception of psychological stress exerted by the other fish, or perception of sounds emitted by the other fish (Colley and Parmentier 2012; Johnston and Dixson 2017; Desrochers et al. 2020). How such social signals are processed in the brain to initiate sex change remains completely unknown in any species of sex-changing fish to our knowledge.

### 12.2.2 POA SEX CHANGE

In anemonefishes, the anatomy of the POA changes to the female phenotype before gonadal sex change and before behavioral sex change occurs. A cell population has been identified in the anemonefish anterior POA that contains roughly twice as many cells in females compared to males, controlling for body size differences (Dodd et al. 2019; Figure 12.3A–D). This cell population is feminized by six months after the initiation of sex change, before

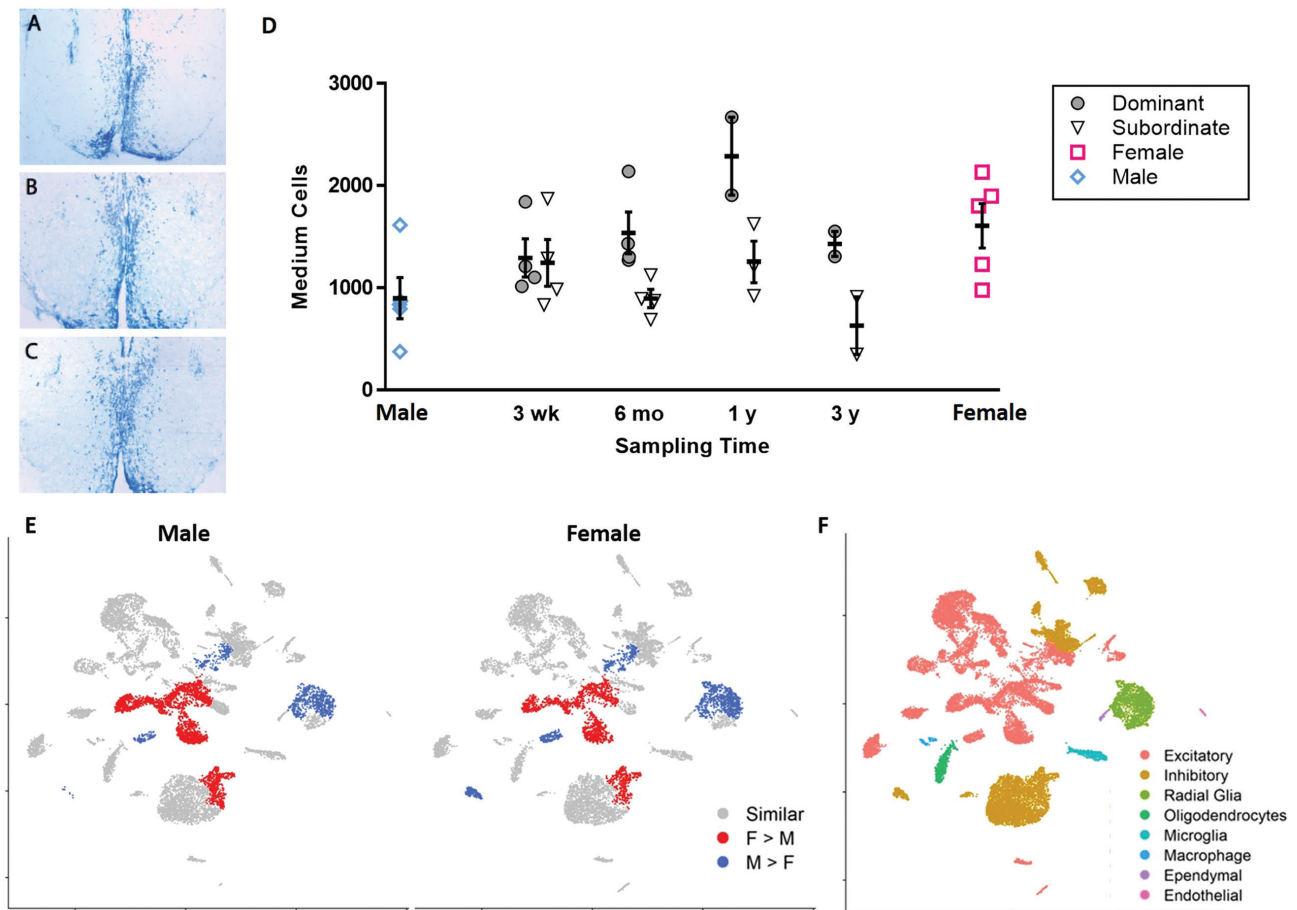
the sex-changing fish displays vitellogenic oocytes and female-typical gonadal sex hormones. A recent analysis of single nuclei RNA sequencing (snRNA-seq) of the POA region from six female and six male *A. ocellaris* revealed a number of different neuron populations that are sexually dimorphic in cell number, including several candidates displaying an increased number of inhibitory neurons in females (Figure 12.3E–F). The anterior POA population is of particular interest as a putative homologue or phenologue of the rodent anteroventral periventricular nucleus (AVPV). The AVPV contains more inhibitory neurons in females compared to males and is crucial in regulating ovarian hormone cycling and ovulation. If functional or developmental similarities can be established between the anemonefish anterior POA and the rodent AVPV, anemonefishes could prove indispensable to the study of vertebrate brain feminization.

### 12.2.3 BEHAVIORAL SEX CHANGE

Some of the earliest published work on anemonefishes described their remarkable sex differences in aggressive and parental behavior (Ross 1978a, 1978b). Females are much more aggressive towards other females as compared to males, defending their territory vigorously from female intruders and reinforcing the social hierarchy within their group using a combination of aggressive displays, charging, and biting. Anemonefish males provide the majority of parental care to developing eggs, tending to them with frequent fanning (to keep water circulating over the eggs and remove debris drifting nearby) and mouthing (to keep nearby algae and other threats to the eggs in check) (see Chapter 15, “Parental Care: Patterns, Proximate, and Ultimate Causes and Consequences”).

#### 12.2.3.1 Sex Change in Aggression

Sexually differentiated aggression in anemonefishes has been well described, but only one published study has actually assessed aggression *during* sex change. Field work with *A. melanopus* assessed aggressive behavior of dominant sex-changing fish towards conspecific female intruders (Godwin 1994b). Fish were tested 15 days after the resident female was removed, and were found to display approximately two-fold greater aggression than a resident male. While an increase in aggression does represent a shift towards more “female-like” behavior, it is important to compare this with the patterns of aggression expected from true females. Earlier fieldwork with *A. melanopus* assessed aggression from resident males and resident females towards a conspecific female intruder and found that females displayed between two- and 12-fold greater aggression than their male partners, depending on the pair and the distance of the intruder from the centre of the territory (Ross 1978a). More recent laboratory work with *A. ocellaris* has found that female residents display approximately five-fold greater aggression, on average, than male residents towards a female intruder (Iwata and Manbo



**FIGURE 12.3** Sexually dimorphic cell populations in *Amphiprion ocellaris* POA region. A–C) Representative sections through the anterior POA from a reproductive male, a sex-changing fish six months after induction, and a reproductive female, respectively. Photos were taken at 50X magnification. D) Total number of medium sized cells in the anterior POA plotted against time-point following induction of sex change with reproductive males (M) on the left and reproductive females (F) on the right. Means are shown as horizontal bars within the scatter plot with standard errors bars in the vertical position. Significant differences were observed between dominant (sex-changing) and subordinate (not sex-changing) at the six-month and one-year time points. None of the subordinate groups differed from males, whereas dominant members from the six-month and one-year time-points displayed significantly greater numbers of medium cells than males. Females were significantly different from six-month subordinate and three-year subordinate groups. E) Approximately 24,000 cellular nuclei from the POA (and surrounding region) of six male and six female *A. ocellaris* were analyzed by single nuclei RNA-sequencing. Each cell is plotted as a point on two multi-dimensional scaling axes such that cells that are farther apart are more distinct in their nuclear transcript profiles. The first two plots show males and females separately and identify the clusters of cells that are sexually dimorphic in number. Red clusters represent cell types that are more numerous in females than males (i.e., had a greater proportion of cells of that type), whereas blue clusters were more numerous in males than females. F) The third plot shows all the nuclei (males and females together) and identifies the major cell types based on multiple canonical cell-type specific markers.

2013). This study also assessed aggression towards male intruders, finding that resident males and females display comparable levels of aggression towards a male intruder, and at a level much lower than the level of aggression seen by females towards females.

Altogether, with regard to conspecific aggression, females are not simply more aggressive. Rather, they are selectively more aggressive towards female conspecifics, and to a degree that is unmatched by males in any context. Recent data suggest that while sex-changing fish display slightly elevated aggression towards both males and females, the levels of aggression displayed towards females are nowhere near those displayed by typical females towards

females. Thus, the aggression phenotype of a sex-changing *A. ocellaris* is neither male nor female. It is unique in that it constitutes moderately elevated indiscriminate aggression towards both sexes. Moreover, as described below, the phenotype of a sex-changing *A. ocellaris* is decidedly male-like when it comes to parental care.

### 12.2.3.2 Sex Change in Parental Care

While female anemonefish spend their time patrolling their territory and preserving its integrity, male anemonefish contribute to the success of the mating pair by picking up parenting duty (Ross, 1978b; DeAngelis et al. 2017, 2018). Unlike aggressive and defensive behavior, which are just

as necessary during sex change as any other time, parental behavior is not expressed under natural conditions for a sex-changing fish, as they will not have offspring. However, recent research shows that even lone non-breeding fish with no parenting experience indiscriminately care for unrelated conspecific eggs in their territory (Phillips et al. 2020). A recent experiment in which sex-changing fish and their partner were given eggs from another spawning pair in the colony, established the sex-changing fish as the primary caregiver of the foster eggs. Sex-changing fish displayed similar levels of foster care as mature males did. The subordinate member of the pair spent significantly less time in the nest and less time caring for the eggs. These data reinforce the notion that sex-changing anemonefish behave more like males than females. Further, even in those pairs in which the dominant sex-changing fish had developed female gonads with vitellogenic oocytes by the time of behavioral assessment, these fish provided male-like parental care. Taken together the data suggest that behavioral sex change occurs after gonadal sex change in *A. ocellaris*.

### 12.2.3.3 Neural Mechanisms of Behavioral Sex Change

General neuroendocrine mechanisms underlying aggression and parental care in anemonefish have been explored, but how such mechanisms are modified during protandrous sex change has not been investigated yet to our knowledge. Isotocin and arginine-vasotocin both play a role in regulating parental care and aggression in anemonefish (DeAngelis et al. 2017, 2020), and estradiol administration increases aggression at least in undifferentiated non-breeder fish (Iwata and Suzuki, 2020). Conspecific aggression (which is highly sexually differentiated, as described earlier) is positively regulated by arginine-vasotocin signalling and is associated with cell activation in the POA and the periventricular nucleus of the posterior tuberculum (Yaeger et al. 2014). Female anemonefish have higher whole-brain aromatase (estradiol synthase) gene expression, and males have higher whole-brain isotocin receptor gene expression (Casas et al. 2016; DeAngelis et al. 2018). These differences in gene expression may play a role in mediating the sexual differentiation of aggressive and parental behavior. Current theories about the regulation of social behaviors emphasize the role of neuropeptides like isotocin and arginine-vasotocin, and possibly also neurosteroids like estradiol, in modulating patterns of correlated cell activation across a network of brain regions involved in social decision making (Johnson and Young 2017). Sex differences in aromatase and isotocin receptor expression may be confined to one or many nodes of the network, and changes in expression levels would be a convenient mechanism by which gonad-independent behavioral sex change may be accomplished.

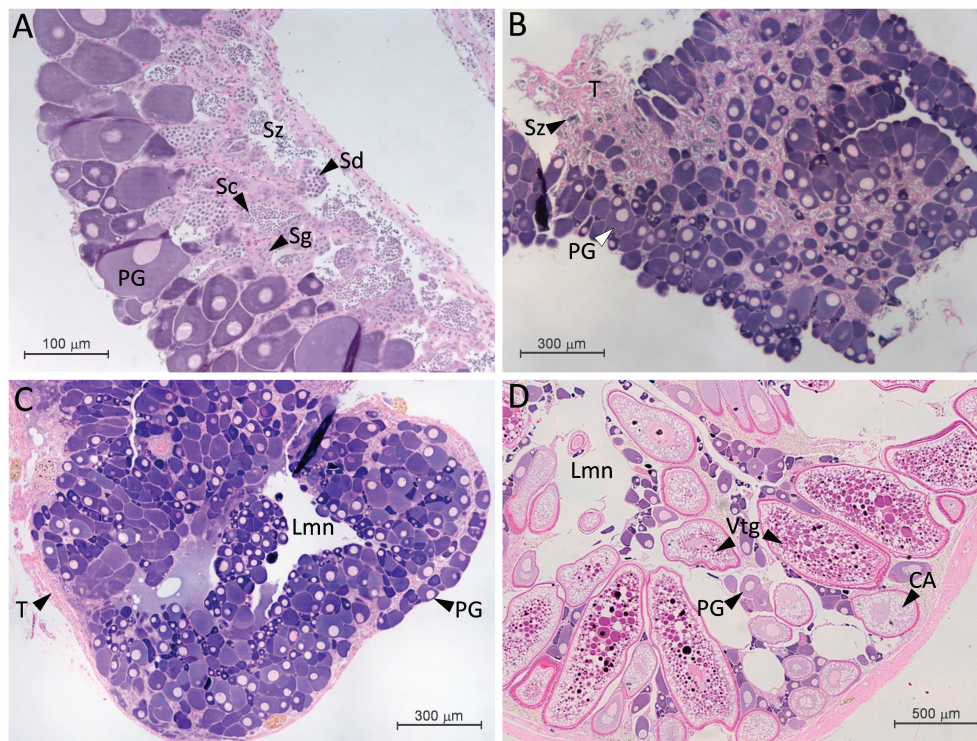
## 12.3 GONADAL SEX CHANGE

The complex genomic response of the brain associated with sex change is subsequently transmitted to the gonads along the hypothalamic-pituitary-gonadal axis. Receptors on the

gonadal tissue receive the hormonal signals that stimulate the corresponding resorption or extension, completing gonadal reorganization (Kobayashi et al. 2009). This process involves a complete restructuring of the gonadal tissue, which can be accomplished in a few weeks or take years, depending on both the social environment and the anemonefish species. The fastest completion of sex change reported in the genus corresponded to *A. bicintus*, with the sex-changing mate laying eggs 26 days after the disappearance of the dominant female (Fricke and Fricke 1977). In several other species, including *A. clarkii*, *A. akallopisos*, and *A. melanopus*, the process lasts several months while in *A. frenatus* it can take up to several years (Fricke and Fricke 1977; Moyer and Nakazono 1978; Hattori 1991; Godwin 1994a). Moreover, in captive conditions, reorganization of the gonads might be delayed for long periods after brain feminization (Dodd et al. 2019). It has been suggested that the timing of feminization might be size-dependent and that sex change is contingent upon the presence of a smaller conspecific to complete the breeding pair (Fricke 1983; Hattori 1991; Casas et al. 2016). Nonetheless, other factors potentially explaining differences within and among anemonefish species have not yet been described.

### 12.3.1 HISTOLOGICAL CHANGES ACROSS SEX CHANGE IN AMPHIPRION

As protandrous species, the primary development in anemonefishes is female while male development is a temporary phase (Figure 12.4). During early gonadal development, immature individuals show mainly ovarian tissue with primary perinucleolus oocytes and no distinguishable spermatogenic cells. Later, in the juvenile phase, ovotestis show primary growth phase oocytes and male germinal cells at different stages of development, including some spermatozoa (Casadevall et al. 2009). As the fish matures into a breeding male, the testicular tissue expands gradually occupying most of the gonadal cavity while the female region is restricted to the periphery. The male stage is characterized by seminiferous tubules with cells at different stages of development, organized in cysts that open up upon completion of spermiogenesis to discharge the spermatozoa into the lobular lumen and, subsequently, the sperm duct. Males only possess oocytes in the primary growth phase (i.e., oogonia, chromatin-nucleolar, and perinucleolar stages), peripherally located (Figure 12.4A). The transition from male to female starts with the formation of an ovarian cavity or lumen and the displacement of the spermatogenic tissue towards the periphery of the gonad. The ovarian tissue is still mainly composed of oocytes in the primary growth phase but cells in the stage of cortical alveoli start to form, indicating the readiness to start vitellogenesis. In the testicular tissue, all the spermatogenic stages (from spermatogonia to spermatozoa) are still present (Figure 12.4B). As sex change progresses, the delimitation of the lumen completes, and the testicular tissue starts to degenerate while the number of oocytes in the stage of cortical alveoli increases significantly



**FIGURE 12.4** Histological sections of the Red Sea anemonefish (*Amphiprion bicinctus*) ovotestis across sex change displaying four different gonadal stages from testis to functional ovary. A) Longitudinal section of a functional male. Ovary and testes occupy half of the gonad each, oocytes are in primary growth stage (PG), while various stages of development can be observed in male germ cells (Sg: Spermatogonia; Sd: Spermatida; Sc: Spermatocyte; Sz: Spermatozoid). B) Transverse section of a specimen at an early transitional stage of sex change. Testicular tissue (T) has reduced and mostly spermatozooids are visible, while other male germ cells are degenerated. Ovary tissue has expanded and only PG oocytes are present. C) Transverse section of an individual at a late transitional stage of sex change (immature female). Testicular tissue (T) is restricted to a small portion in the periphery of the gonad and highly degenerated while no germ cells are detected. The lumen of the ovary (Lmn) is visible, but still only PG oocytes are present. D) Transverse section of a mature female. Oocytes in secondary growth stages are visible (CA: cortical alveoli; Vtg: vitellogenic oocyte). Figure originally published in Casas et al. (2016).

(Figure 12.4C). The transition from male to female is completed when oocytes begin to mature and the testicular tissue of the ovotestis is resorbed. The full degeneration of the testicular tissue indicates that the sex change is irreversible (Casas et al. 2016). The mature female gonad is characterized by well-developed ovarian tissue with oocytes of all stages (chromatin-nucleolar, perinucleolar, and cortical alveoli, vitellogenic stages, mature and atretic oocytes) and asynchronous ovarian development (Figure 12.4D).

### 12.3.2 ENDOCRINE REGULATION OF SEX CHANGE: SEX STEROIDS

Sex change was first described in anemonefishes in the late 1970s (Fricke and Fricke 1977) and 16 more years were necessary for the characterization of the first key players involved in the process (Godwin and Thomas 1993). Significant progress has been made since, but it was not until recently that a pioneering wide-genome study of sex change in the Red Sea anemonefish provided insights into the global molecular mechanism orchestrating social sex change and gonadal restructuring (Casas et al. 2016; Casas et al. 2018).

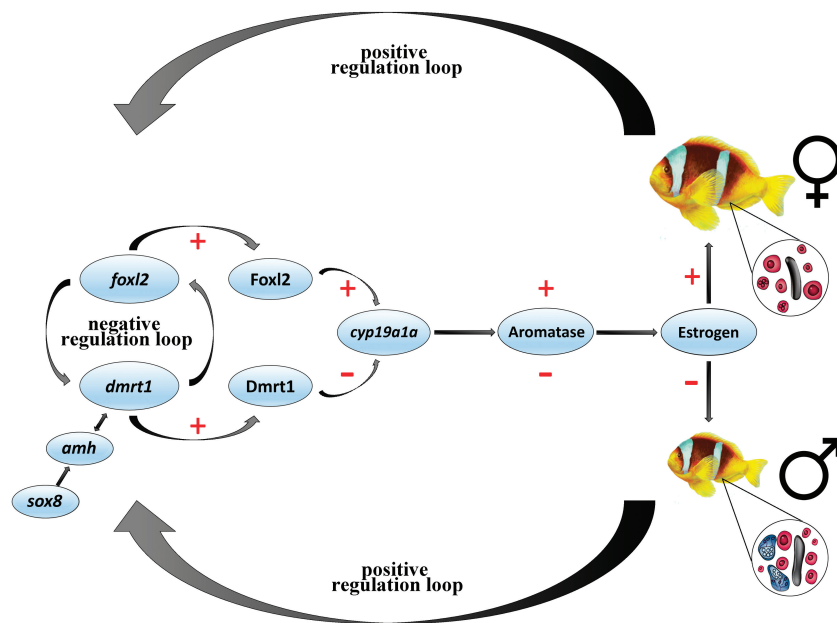
The sexual identity depends on the balance between gonadal estrogen and androgen production, which promote ovarian and testicular function, respectively, in vertebrates. In teleosts, the major relevant steroids are  $17\beta$ -estradiol (E2) and 11-ketotestosterone (11-KT). Their production depends on the bioconversion of testosterone (T), which acts as a prohormone in fish, via two opposing pathways (Frisch 2004; Guiguen et al. 2010). In all sex-changing fishes, pronounced shifts in the balance of sex steroids occur across sex change and marked sex-biased levels characterize males and females. In anemonefishes specifically, a sharp drop in 11-KT levels precedes testis degeneration and is followed by a gradual increase in E2 production accompanying the progression of sex change (Godwin and Thomas 1993). High 11-KT levels and low E2 levels are present in functional males, whereas the opposite trends characterize functional females (Nakamura et al. 1994; Kobayashi et al. 2010; Mills et al. 2018). Moreover, treatment of differentiating juveniles with exogenous E2 prevents the differentiation of testicular cells forming exclusively ovarian tissues (Miura et al. 2013).

### 12.3.3 MOLECULAR PATHWAYS UNDERLYING GONADAL SEX CHANGE

At the molecular level, the maintenance of the sexual identity in anemonefishes is dependent on male and female pathways, well conserved in all vertebrates, acting antagonistically (Figure 12.5). Thus, the simultaneous activation of the appropriate sex-specific network and the suppression of the sex-opposing network actively regulate gonadal fate (Capel 2017). One of the best-known players in the feminizing gene network is the *cyp19a1a* gene, which encodes the gonadal aromatase and controls the sex steroid balance. This enzyme catalyzes the biosynthesis of estrogens from androgens and is essential for the maintenance of ovarian function in vertebrates (Guiguen et al. 2010). The ovarian aromatase gene has been proposed to play a central role in sex change in anemonefishes acting as the potential switch of the female pathway by causing the rise of estrogen production and the concomitant collapse of the male network. The expression of this gene shows a sharp upregulation in transitional females and remains overexpressed until the completion of sex change in *A. bicinctus* (Casas et al. 2016). The administration of an aromatase inhibitor to functional females of *A. clarkii* causes their masculinization, resulting in active spermatogenic germ cells in the ovarian tissue (Nakamura et al. 2015). Another key member of the feminizing network in vertebrates, including mammals and fish, is the forkhead transcriptional factor L2 (*foxl2*) (Uhlenhaut et al. 2009; Siegfried 2010; Georges et al. 2014). It is essential for ovarian differentiation and maintenance in fish since it regulates

aromatase expression and hence, the synthesis of estrogen. Since *foxl2* can upregulate aromatase and estrogens upregulate *foxl2* in fish, a positive feedback loop between both genes has been proposed (Wang et al. 2007; Yamaguchi et al. 2007; Guiguen et al. 2010). In anemonefishes, is proposed to be pivotal for the activation of the female pathway driving the gonadal transformation from testis to ovary during sex change. Accordingly, the spatio-temporal expression profiles of both genes across sex change in *Amphiprion* are highly correlated showing a marked downregulation in male stages (Casas and Saborido-Rey 2021). Additionally, several genes required for ovarian steroid production (the steroidogenic acute regulatory protein [*Star*], the enzyme estradiol 17 $\beta$ -dehydrogenase 1 [*hsd17b1*]), have also been proposed as relevant members of the feminizing network in anemonefishes. Their expression is upregulated in mature females and correlates significantly with the regression of the testis and the development of the ovary.

The opposing-male promoting network consists of several widely conserved components of sex determination, testicular differentiation, and spermatogenesis in teleosts. The doublesex and mab-3 related transcription factor 1 (*dmrt1*) interacts antagonistically with *foxl2* to regulate aromatase expression and thus, estrogen production (Matson et al. 2011; Li et al. 2013). Gene *dmrt1* is not only fundamental in the differentiation and maintenance of the gonadal male identity across vertebrates but also for the inhibition of the female pathway (Matson and Zarkower 2012). The expression profile of *dmrt1* in anemonefishes and other protandrous fish shows a steady decline across



**FIGURE 12.5** Model of the molecular mechanism underlying gonadal sex change in anemonefish. When sex change is triggered, the hypothalamic-pituitary-gonadal axis communicates the brain response to the gonad, which induces a rapid spike of aromatase production. This produces the upregulation of *foxl2* and the synthesis of steroid hormones tipping the sex steroid balance towards the estrogen. At the same time, a negative feedback loop between *foxl2* and *dmrt1* downregulates the male pathway genes shutting down the male network, allowing gonad remodelling. Gene names are written with lowercase italics while, for protein names, non-italic and the first letter in uppercase are used, following the zebrafish nomenclature convention. Figure modified from Casas et al. (2016).



sex change, paralleling the regression of the testis (He et al. 2003; Casas et al. 2016; Zhang et al. 2019). A second essential member of the male pathway in fish is the anti-Müllerian hormone (*amh*) gene (Pfennig et al. 2015; Adolfi et al. 2019). The exact function of *amh* in fish remains unclear but it has been proposed as a candidate to downregulate aromatase (Wang and Orban 2007). In anemonefish and the protogynous black porgy, a steady decrease in *amh* expression values is observed throughout sex change, following closely the expression of *dmrt1* (Wu et al. 2010; Zhang et al. 2019; Casas and Saborido-Rey 2021). The activity of *amh* is regulated by *sox* genes, members of a large family of transcription factors that encode key mediators of testis determination and male fertility maintenance in mammals (Jiang et al. 2013). The most prominent member of this family is *sox9* as it is necessary and sufficient to induce testis differentiation in various vertebrates (Vining et al. 2021). Although its function is poorly understood in fish, *sox9* has been reported as either male-restricted or strongly male-biased in at least ten hermaphroditic species, including protogynous and protandrous fish (Manousaki et al. 2014; Liu et al. 2015; Tsakogiannis et al. 2018, 2019). However, studies in anemonefish do not support an important role of this gene in sex change. An alternative family member, *sox8*, has been proposed as an important determinant for the maintenance of testis cell identity (Casas et al. 2016). This gene is known to be essential for male fertility maintenance in mammals and functions redundantly with *sox9* in the maintenance of spermatogenesis (Barrionuevo et al. 2016). In anemonefish, expression of *sox8* is upregulated in males and steadily decreases as sex change progresses (Casas et al. 2016).

The molecular mechanism described here involves a complex regulatory loop combining steroid hormonal activity with transcriptional regulation of well-conserved genes among vertebrates and teleosts. It has been proposed to be common across sequential hermaphrodites, with protandrous species sharing a mirrored mechanism with protogynous sex-changers (Casas and Saborido-Rey 2021). However, the precise organization of the gene network directing sex reprogramming has not been elucidated in anemonefishes or any other hermaphrodite fish species yet. Similarly, few studies have addressed the epigenetic processes mediating sex change in fish and a detailed mechanism is still lacking.

## 12.4 CONCLUSION

Anemonefishes are now established as the best-studied group of protandrous fishes with socially controlled sex change, as evidenced by the rich literature surveyed in this book. They are well suited to the study of protandrous sex change, in both its molecular mechanisms and the peculiar ecological factors that define the anemonefish subfamily and drove the emergence of their particular mode of sex change. There is no doubt that future research in this model will continue to surprise us and enrich our understanding

of sex change and vertebrate sexual development more generally.

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