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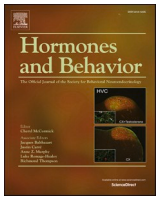

Stable and persistent male-like behavior during male-to-female sex change in the common clownfish *Amphiprion ocellaris*

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ABSTRACT

Many fish species exhibit natural sex change as part of their life, providing unique opportunities to study sexually-differentiated social behaviors and their plasticity. Past research has shown that behavioral sex change in the female-to-male (protogynous) direction occurs rapidly and well before gonadal sex change. However, little is known about the timecourse of behavioral sex change in male-to-female (protandrous) sex-changing species, limiting our ability to compare patterns of behavioral sex change across species and identify conserved or divergent underlying mechanisms. Using the protandrous sex changing anemonefish *Amphiprion ocellaris*, we assessed behavior (aggression and parental care) and hormones (estradiol and 11-ketotestosterone) in fish over six months of sex change, and compared those fish against their non-changing partners as well as control males and females. Contrary to expectations, we found that sex-changing fish displayed behavior that was persistently male-like, and that their behavior did not become progressively female-like as sex change progressed. Hormones shifted to an intermediate profile between males and females and remained stable until gonads changed. These results support a new perspective that the timecourse for protandrous sex change in anemonefish is completely distinct from other well-established models, such that behavioral sex change does not occur until after gonadal sex change is complete, and that sex-changing fish have a stable and unique behavioral and hormonal phenotype that is distinct from a male-typical or female-typical phenotype. The results also identify aspects of sex change that may fundamentally differ between protandrous and protogynous modes, motivating further research into these remarkable examples of phenotypic plasticity.

1. Introduction

Social behaviors like courtship, aggression, and parental care are differentiated by sex across vertebrate species. Gonadal estrogens and androgens play a central role in regulating these sexually differentiated behaviors (Bridges, 2015; Hashikawa et al., 2018; McCarthy and Arnold, 2011; Rogers and Bales, 2019; Schulz and Sisk, 2016). Socially-controlled sex change, a characteristic of a number of families of marine fish, provides a unique opportunity to study sexually-differentiated behaviors and their plasticity (Casas and Saborido-Rey, 2021; Erisman et al., 2013; Kuwamura et al., 2020; Lamm et al., 2015; Prim et al., 2022). In such fish sex is determined by social status. This means that fish will spend part of their lives as one sex (e.g. a male with functional testes and male-like behavior), 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 displaying female-like behavior). Different species exhibit either protogynous (female-to-male), protandrous (male-to-female), or bidirectional sex change. Socially-controlled sex change provides a natural context in which to study dramatic plasticity of sociosexual behaviors, and descriptions of the timecourse of behavioral sex change relative to other facets of sex change (i.e. gonadal or hormonal sex change) provide important clues to the possible underlying mechanisms (Black et al., 2005a; Lamm et al., 2015; Prim et al., 2022).

Behavioral sex change has been best-studied in protogynous species where it has two major qualities: it is 1) rapid and 2) independent of gonadal steroid hormones. Rapid behavioral sex change in these species entails the emergence of male-like courtship behavior and aggressive behavior within 24 h of sex change initiation by dominance ascent. This has been described during protogynous sex change in many species,

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including many wrasses across multiple genera (Godwin et al., 1996; Hoffman et al., 1985; Nakashima et al., 2000; Sakai et al., 2002; Warner and Swearer, 1991), an angelfish (Sakai et al., 2003), a basslet (Shapiro, 1981), and multiple gobies (Black et al., 2005b; Grober and Sunobe, 1996; Reavis and Grober, 1999; Rodgers et al., 2007, 2005). The onset of behavioral sex change in such a short timeframe implies independence of gonadal hormones, as it takes two to three weeks for gonadal and hormonal sex change to occur (Reavis and Grober, 1999; Todd et al., 2019; Warner and Swearer, 1991). Moreover, complete removal of the gonads does not interfere with behavioral sex change in the Bluehead wrasse (Godwin et al., 1996). And while behaviors associated with the new sex do newly appear within 24 h, the actual frequency of the behaviors does not rise to fully male-like levels until four or five days into sex change (Godwin et al., 1996; Reavis and Grober, 1999), suggesting a rapid initial shift followed by gradual reinforcement of the underlying neuroendocrine mechanisms. The mechanisms implicated in this dramatic example of sociosexual behavioral plasticity include brain-derived estrogens and the neuropeptides oxytocin and vasotocin responding rapidly to changes in social status (Black et al., 2011; Black et al., 2005a; Black et al., 2004; Godwin et al., 2000; Grober and Sunobe, 1996; Lema et al., 2012; Marsh-Hunkin et al., 2013; Prim et al., 2022; Semsar et al., 2001; Semsar and Godwin, 2004; Semsar and Godwin, 2003) (for a note on nomenclature for these neuropeptides, see (Theofanopoulou et al., 2021)). Locally-synthesized estradiol and nonapeptides are major mediators of rapid social behavioral plasticity across vertebrate species, as well (Cornil, 2018; Cornil and Charlier, 2010; Goodson and Thompson, 2010; Trainor et al., 2006).

However, the timecourse of behavioral sex change in protandrous species is not well understood, and thus neither are the underlying mechanisms. Anemonefish (clownfish) are among the best-studied protandrous species, in part due to their unique life history and patterns of sexually differentiated behavior (DeAngelis et al., 2020; DeAngelis et al., 2017; DeAngelis and Rhodes, 2016; Godwin, 1994a; Gonzalez et al., 2021; Iwata and Manbo, 2013; Ross, 1978a, 1978b; Wong et al., 2013). Female anemonefish are dominant in the social hierarchy and are highly aggressive, while males are less aggressive and provide most of the parental care toward offspring. In anemonefish, sex change is induced in a reproductive male when its dominant female mate is removed, or when two reproductive males are paired together and one male (usually the larger) establishes dominance. Only one study has assessed behavior during sex change in anemonefish, finding that sex-changing fish display 2 to 3-fold greater aggression toward a conspecific female intruder than control males (Godwin, 1994a). While this clearly demonstrates a departure from a male-like magnitude of aggression, it is not sufficient evidence to conclude a sex-changed or female-like pattern of aggression. This is because female anemonefish are not simply more aggressive than males. Instead, they are selectively more aggressive toward female conspecifics than they are toward male conspecifics. Female anemonefish display an average 5-fold greater aggression than male anemonefish when faced with a female intruder, while female and male anemonefish display comparable levels of aggression toward a male intruder (Iwata and Manbo, 2013). And just as female anemonefish display a sex-specific pattern of aggression, male anemonefish display a sex-specific degree of parental care that is greater than what a female will display (DeAngelis and Rhodes, 2016). Female anemonefish do not display male-like levels of parental care even when their male partner is handicapped or removed (Barbasch et al., 2021; Phillips et al., 2020). Investigating the timecourse and mechanisms underlying behavioral sex change in anemonefish has potential to illuminate our understanding of the neurobiology underlying sex-specific patterns of aggression (Hashikawa et al., 2018; Yaeger et al., 2014) and male parental care (DeAngelis et al., 2017; Rogers and Bales, 2019).

Other aspects of protandrous sex change in anemonefish follow a very different timecourse compared to protogynous models, which further motivates a closer investigation of the timecourse of behavioral sex change in anemonefish. While gonadal sex change in protogynous species is reliably completed within two to three weeks (Reavis and Grober, 1999; Todd et al., 2019; Warner and Swearer, 1991), in anemonefish the shortest reported timespans are within one to two months (Fricke and Fricke, 1977; Godwin, 1994b). Still, one to two months is relatively rare, as the majority of anemonefish across numerous reports did not complete gonadal sex change until four to six months or longer (Casas et al., 2016; Dodd et al., 2019; Godwin, 1994b; Moyer and Nakazono, 1978). Sex-changed gonadal hormone secretion occurs in anemonefish in concert with gonadal sex change (Dodd et al., 2019; Godwin, 1994b; Godwin and Thomas, 1993). While gonadal and hormonal sex change has a highly variable timecourse in anemonefish, a sex difference in the preoptic area of the hypothalamus is reliably sex-changed within six months after the initiation of sex change (Dodd et al., 2019). If behavioral sex change in anemonefish is indeed gonad-independent (as in protogynous species) it may instead be related to sexual differentiation of the preoptic area or other brain regions.

In this study we sought to evaluate the timecourse for behavioral sex change in an iconic and phylogenetically basal anemonefish species, the common clownfish *Amphiprion ocellaris*. We assessed aggressive behavior, parental care behavior, and circulating sex hormones throughout six months of sex change and tested whether sex-changing fish were more similar to control males or females on these measures. We expected circulating sex steroids to remain male-like throughout sex change until vitellogenic oocytes were present in the gonad, consistent with previous work (Dodd et al., 2019). We also expected behavior in sex-changing fish to be progressively more female-like toward later timepoints, changing roughly in concert with the timecourse of sex change in the preoptic area (Dodd et al., 2019).

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"). Prior to the experiment, each of these 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 multiple times per week. Experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Fish were housed in twenty-gallon tall (24″ × 12″ × 16″) aquarium tanks integrated with a central circulating filtration system. Conditions mimicked the *A. ocellaris* natural environment (system water temperature range 26–28 °C, pH range of 8.0–8.4, specific gravity of 1.026, 2:12 photoperiod with lights on at 0700 and off at 1900 h). Fish were fed twice daily with Reef Nutrition TDO Chroma Boost pellets.

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 fight 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. Total body length (from the tip of the snout to the end of the tail) was measured to the nearest millimeter, and body mass was measured to the nearest tenth of a gram. 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 regular aggressive displays (chasing, biting, lunging at subordinate fish) (Table 1). Subordinate fish will not aggress in kind, and instead will produce submissive displays (i.e. fleeing when chased, trembling) (DeAngelis et al., 2020; Fricke and Fricke, 1977; Gonzalez et al., 2021; Iwata and Manbo, 2013; Wong et al., 2013). These behaviors are well described in previous work in anemonefish (DeAngelis et al., 2020; Gonzalez et al., 2021; Iwata and Manbo, 2013; Wong et al., 2013), and are summarized in Table 1. 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. 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 typically 20–30 % longer and approximately 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 (Fig. 2), consistent with dominance establishment and thus sex change.

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. The 30 pairs were assigned to one of six timepoints for behavioral testing and blood hormone assessment: either 6, 10, 14, 18, 22, or 26 weeks after pairs were established (n = 5 dominant and n = 5 subordinate fish per timepoint). Unfortunately, one pair of fish assigned to the week 18 timepoint had to be removed from the study because the dominant fish died. Two fish happened to have completely changed sex by the time that behaviors and hormones were assessed, and these fish and their partners were grouped separately for analyses (see below, "2.3. Terms and Definitions Regarding Sex Change"). One of these fish was in the 18 week timepoint, and the other was in the 26 week timepoint. Altogether this 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 twenty-six weeks (six months) was chosen because previous work has shown that at least one sexually-differentiated cell population in the anemonefish brain is completely sex-changed within six months (Dodd et al., 2019).

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. Only four control pairs were available for this study due to resource limitations, as maintaining continuously breeding male-female pairs in captivity is not trivial. To improve the sample size for control males and females and thus the quality of our analysis we have included additional control male and female datapoints from a previously published report (DeAngelis and Rhodes, 2016) for parental care and steroid hormones (see sections "2.4.2. Parental Care Assay and "2.5. Steroid Hormone Immunoassays").

Table 1
Behaviors and their descriptions. The same behaviors were recorded during encounters with reproductive males and females. Behaviors and their descriptions are consistent with previous work where aggression and parental behaviors were measured.

Aggressive behaviors	
Bite	One fish darts at the other making contact with its mouth
Lunge	One fish darts at the other without making contact
Chase	One fish chases the other over a distance greater than one chaser's body length
Clash	Both fish dart at one another rapidly and simultaneously after facing off for a moment

Parental behaviors	
Nip	Mouthing near the eggs to clear the substrate
Fan	Using the pectoral fins or a full-body shake over the eggs to circulate water

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 within the past three months. Control male and female fish used in this study, as well as stimulus fish used for behavioral assays, 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, 1994b). Based on these criteria, we happened to find 2 out of 29 (7 %) dominant fish in this study had completed gonadal sex change by the time of behavioral assessment and tissue collection. One of these fish was in the 18 week timepoint, and one was in the 26 week timepoint. 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 blood 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. Behavioral tasks

Behavioral testing was carried out over 4 consecutive days beginning 6, 10, 14, 18, 22, or 26 weeks after the initiation of sex change for experimental pairs, as described above. For control male-female pairs, behavioral testing was carried out at a random timepoint in their spawning cycle. The behavioral testing timeline is summarized in Fig. 1. As in similar past work (Dodd et al., 2019), a between-subjects design was used so that we could examine the gonads for presence of vitellogenic oocytes as an indicator of gonadal sex change immediately after behavioral assessment was complete, which requires euthanasia. Behaviors were assessed daily one after the other to balance two competing considerations: the desire to assess behaviors and hormones as close in time as possible to one another, and the desire to limit the influence of being subjected to one assay influencing the outcome of the next. It is still possible that 24 h was not sufficient to "wash out" possible winner/loser effects from one aggression assay to the next, or that being subject to the behavioral assays could to some degree influence hormone values. This is a limitation of the study design accepted in order to allow for all behaviors and hormones to be assessed close together in time. All behavioral tasks were recorded on video and graded using BORIS event-logging software (Friard and Gamba, 2016).

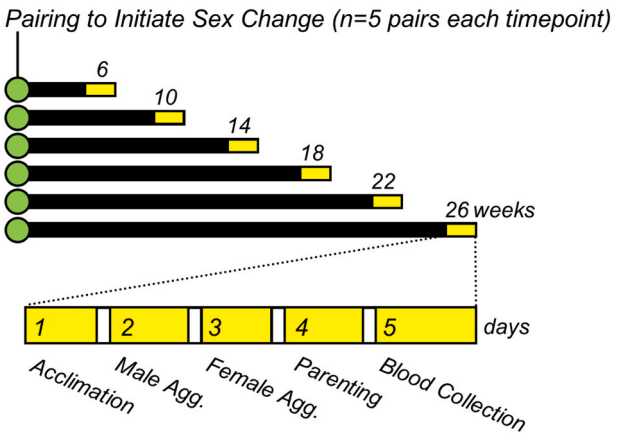

Fig. 1. Summary of study design. Thirty dominant-subordinate pairs were generated from sixty male *A. ocellaris*. Pairs were assigned to one of six timepoints for behavioral and hormone assessment (n = 5 pairs per timepoint). The behavioral and hormone assessment took place over five days, beginning with an acclimation day, followed by aggression assays, parental care assays, and blood collection. Control males and females (n = 4) also underwent behavioral and hormone assessment.

2.4.1. Aggression assay

A novel aggression assay was used to discriminate between male-typical and female-typical patterns of aggression. The dominant or subordinate fish was removed from its home tank and placed in an observation tank. Then, a novel male or female fish was introduced to the observation tank and the fish were allowed to interact freely for 15 min. In pilot experiments we paired male and female fish together in this way (unpublished) and found that performance on this assay was highly sexually differentiated. Fish of either sex displayed greater aggression toward a sex-matched fish than they did toward an opposite-sex fish, and sex-matched female encounters evoked a greater magnitude of aggression than sex-matched male encounters. In this way the assay provides a clear litmus test for behavioral sex with respect to aggression.

On the first day of behavioral assessment all fish were acclimated to the ten-gallon observation tank to be used for aggressive behavior assays on days two and three (Fig. 1). Fish were given two 30-minute acclimation sessions, one between 1000 and 1200 h and another between 1400 and 1600 h. Video cameras were set in front of the tanks during acclimation.

On day two of behavioral assessment all fish were assessed for aggressive behavior with novel males. From each tank the dominant and subordinate fish (or, for control pairs, the female and male fish) were placed in separate ten-gallon tanks that they had been acclimated to the day before and video recording began. Stimulus male fish were chosen from breeding pairs in our colony and placed into each tank with the dominant or subordinate fish. The experimenter left the room and fish were allowed to interact freely for 15 min, after which fish were returned to their home tanks. Later the same day, the dominant and subordinate fish were returned to the ten-gallon tanks for another 15-minute encounter with another male from the colony. In this second encounter the dominant and subordinate fish from each tank were placed with the male that their tankmate saw in the first encounter. In this way, each dominant-subordinate pair encountered the same two stimulus fish. The first encounter of the day took place between 1000 and 1200 h and the second encounter took place between 1400 and 1600 h. Aggressive behaviors (biting, chasing, lunging) from both the experimental fish and the stimulus male were counted during each 15-minute encounter. These behaviors are well described in previous work in anemonefish (DeAngelis et al., 2020; Gonzalez et al., 2021; Iwata and Manbo, 2013; Wong et al., 2013), and are summarized in Table 1. In many interactions there were instances of aggressive behavior where the focal fish and the stimulus fish simultaneously faced one another then rapidly clashed together, such that we were not able to confidently determine which fish was the aggressor for that particular behavior. We coded these behaviors as "clashes" separate from bites, chases, or lunges (Table 1). On the next day (day three) all fish were assessed for aggressive behavior against novel females. The approach was identical to that described above for aggressive behavior against novel males, instead using females from the colony.

For each experimental fish a male-oriented aggression score and a female-oriented aggression score were calculated. The total number of aggressive acts (clashes, bites, lunges) displayed by the experimental fish toward the male stimulus fish in each encounter was averaged to produce the male-oriented aggression score, and likewise for the female-oriented aggression score. In addition, a female:male (F:M) aggression ratio was calculated for each fish as the female-oriented aggression score divided by the male-oriented aggression score. To avoid issues with 0 values in the denominator we added a small constant value of 0.5 to every aggression score, then calculated the F:M aggression ratio for each fish. The F:M aggression ratio was then $\log_{10}$-transformed so that the value would capture the direction and magnitude of the aggression bias toward one sex or the other, centered at zero. By this measure a more negative value reflects a stronger bias toward male-oriented aggression (a more male-typical pattern of behavior) and a more positive value reflects a stronger bias toward female-oriented aggression (a more female-typical pattern of behavior), with a value of 0 indicating no bias.

2.4.2. Parental care assay

The parental care assay used here was similar to previous work describing parental care in anemonefish (DeAngelis et al., 2020; DeAngelis et al., 2017; DeAngelis and Rhodes, 2016; Phillips et al., 2020), and it takes advantage of the tendency for anemonefish to indiscriminately care for unrelated eggs when they are placed in their home tank (Phillips et al., 2020). Parental care assessment took place the day after female aggression assessment (Fig. 1). A video camera was placed in front of the tank for 1 h before the parental care assessment so that fish would acclimate to its presence. The terra-cotta pot serving as the pair's nest site was then removed from the tank and replaced with a pot from another tank in our colony containing a clutch of fertilized eggs. Eggs were between 4 and 6 days of age, and the mean ± standard deviation for number of eggs in a clutch was 725 ± 241. Video recording began when the eggs were placed in the tank, then experimenters left the room and fish were allowed to interact with the eggs freely for 15 min. The first 10 min of video was discarded, and the remaining 5 min was graded by a trained experimenter. Parental behaviors in anemonefish include "nipping" and "fanning", which are well described in previous work (DeAngelis et al., 2020; DeAngelis et al., 2017; DeAngelis and Rhodes, 2016; Phillips et al., 2020), and are summarized in Table 1. The number of eggs in the pot was counted before and after each parenting session to confirm that there was no substantial egg loss during the session. Egg care assessment took place between 1000 and 1200 h.

Given that only four male-female pairs were available for this study due to resource limitations, we included additional male and female datapoints from our previously published work using a similar parental care assay (DeAngelis and Rhodes, 2016). In the published study the parental care behavior of five regularly spawning male-female pairs was assessed during three separate spawning events, providing a total of n = 15 additional male and n = 15 additional female datapoints. The parental care behaviors (nips, fans, time spent in the nest) were quantified during a 5-min period as in the present study. The differences between the past study and the present study are as follows. First, the eggs in the past study were related to the fish caring for them, while in the present study the eggs are unrelated. We do not expect this to influence the analysis as *A. ocellaris* will care for unrelated eggs to the same degree as for related eggs (Phillips et al., 2020). Second, the males and females used in the past study were spawning once every month, while the males and females used in the present study spawned a minimum of once within the past three months. In the figure for these data

(Fig. 5) the datapoints from the past study are denoted by a different point shape making them easily discernible. For each fish a parenting score was calculated by adding together the number of nips and fans displayed by each fish during the 5 min behavior quantification period. We also calculated the amount of time that the fish spent in the nest near the eggs over the 5 min period, as in past work (DeAngelis et al., 2020; DeAngelis et al., 2017; DeAngelis and Rhodes, 2016; Phillips et al., 2020).

2.5. Steroid hormone immunoassays

Circulating estradiol (E2) and 11-ketotestosterone (11KT) were assessed in blood plasma using enzyme-linked immunoassays (ELISAs). Blood was collected 24 h after completion of the parental care assay between 1000 and 1200 h. Samples were collected from the caudal vein with a butterfly needle treated with heparinized 0.9 % saline. Samples were kept on ice, then centrifuged (1250 rcf for 15 min at 4 °C) and plasma supernatant was kept at −80 °C until hormone immunoassays. E2 and 11KT concentrations in plasma were assessed using commercial enzyme-linked immunoassay kits (E2, Calbiotech; 11KT, Cayman Chemical). These kits have been validated in *A. ocellaris* (DeAngelis and Rhodes, 2016). Plasma samples were diluted 1:30 in assay buffer prior to analysis following manufacturer protocol. All samples were run in duplicate. Absorbance was read using an Epoch Microplate Spectrophotometer (BioTek Instruments) at manufacturer-recommended wavelength (412 nm for 11KT, 450 nm for E2).

Blood was collected after the completion of behavioral testing, instead of before, because the small size of *A. ocellaris* makes blood collection an invasive procedure that could interfere with later behavioral measurements. It is possible that the parental care assay 24 h prior to blood collection, or the aggression assays 48–72 h prior, affected the hormone values for these fish. Fish were euthanized immediately after blood collection to allow for inspection of the gonads for the presence of vitellogenic oocytes, indicating gonadal sex change.

Blood collection was unsuccessful for one subordinate fish at the 14-week timepoint, so this fish was omitted from hormone analysis. Blood collection from one control female fish was also unsuccessful, and for another control female only enough blood was collected to assay E2. This left only $n = 3$ control females from this study providing E2 values and $n = 2$ control females providing 11KT values. Because this left us with few control female datapoints, and because we had few control males and females available to begin with, we included additional male and female datapoints from previously published work as described earlier for parental care (DeAngelis and Rhodes, 2016). In the published study blood was collected from five regularly spawning male-female pairs from three separate days after eggs were laid (day 0, day 3, and day 6), as well as during the period between spawning events. In this past study no effect of day was observed on 11KT in either males or females, and an effect of day on E2 was only seen in females such that E2 tended to be higher on days 3 and 6 after spawning (DeAngelis and Rhodes, 2016). These data were collected following the same protocol as the current study with one exception: in the past study samples were spun for 15 min at 1600 rcf, while in the present study samples were spun for 15 min at 1250 rcf. Otherwise blood collection, handling, and plasma dilution was the same, the same kits were used, and plates were read at the same wavelengths on the exact same plate reader. The only other experimental difference is that fish in the previous study had not undergone behavioral assays in the days prior to blood collection. The past study contributes an additional $n = 16$ male and $n = 19$ female datapoints for E2, and $n = 15$ male and $n = 13$ female datapoints for 11KT. In the figure for these data (Fig. 3) the datapoints from the past study are denoted by a different point shape. Hormone values collected from males and females in the previous study are more extreme than those collected in the present study, as two-tailed independent-samples *t*-tests revealed that for each sex and each hormone the fish significantly differed between the two studies (all $p < 0.05$). In the previous study, fish were actively spawning and blood was taken either during the spawning period or within 3–4 days of its completion. In the present study, fish had last spawned one to two months prior to the study. Thus, the differences in hormone values between control fish from the previous and present study may be related to how recently/regularly the pairs were spawning. Control fish from the previous and present study were still pooled for analyses, as this served the purpose of comparing sex-changing fish to control reference points that reflected a range of values expected across reproductive contexts. Circulating E2 and 11KT concentrations were compared among fish, as well as their ratio of E2:11KT calculated by dividing the E2 concentration by the 11KT concentration (both in pg/mL) for each fish.

2.6. Statistical analysis

Statistical analyses were conducted using R (4.1.0). $p < 0.05$ was considered statistically significant for all analyses. To confirm that body mass and body length were indeed different at the time of pairing we applied simple one-tailed *t*-tests for initial body length and body mass. For t-test effect sizes, Cohen's d was calculated as: $d = t\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}$. To confirm that dominant fish grew at a greater rate than subordinate fish (see "2.2. Initiation of Sex Change") we fit a linear mixed effect model for change in body length and change in body mass. The model was fit using function lmer() from R package "lme4" (Bates et al., 2015). 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 function Anova() from R package "car" was used to calculate test statistics and *p*-values for these models. Similar linear mixed effects models were fit for all hormone values (E2, 11KT, and E2:11KT ratio), aggression values (aggression toward females, aggression toward males, and F:M aggression ratio), and parenting values (egg care acts, time in nest) to test for differences between dominant and subordinate fish and how the measures changed over time. In all of these models the new female and new male fish were omitted, leaving only dominant and subordinate fish.

Then, to test how dominant sex-changing fish overall compared to control males and females as well as their subordinate tank mates and the new males and new females on each of these outcome measures, we collapsed dominant and subordinate fish across timepoints and applied one-way ANOVAs followed by Fisher's LSD post-hoc when appropriate. For these analyses the single factor had six levels (dominant, subordinate, new female, new male, female, male). For all linear model effect sizes, partial eta squared (η_p^2) was calculated as: $\eta_p^2 = \frac{SS_{effect}}{SS_{effect}+SS_{error}}$ (Lakens, 2013). In order to generate sums of squares for η_p^2 calculation, we fit a linear model omitting the random effect of tank. Normality was assessed based on diagnostic plots (Q-Q plot and histogram of residuals) and residual 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 the following transformations were necessary before analysis: hormone values (E2, 11KT, and E2:11KT ratio) were all $\log_{10}$ transformed, aggression scores (toward males, toward females, and F:M aggression ratio) were all cube-root transformed, and the number of parental care acts was square root transformed. No other transformations were applied.

3. Results

3.1. Body size change. Larger fish became dominant and displayed increased body growth consistent with dominance and sex change

One-tailed *t*-tests confirmed that, at the time of pairing, fish that would become dominant were significantly larger than fish that would become subordinate in terms of body length ($t_{56} = 9.39$, $p < 0.001$, $d = 2.46$) and in terms of body mass ($t_{56} = 7.38$, $p < 0.001$, $d = 1.94$)

(Fig. 2A, B). On average, the dominant fish in each pair was 15 ± 3 % longer and 51 ± 18 % more massive (mean ± standard deviation) than its subordinate partner.

Dominant fish are expected to grow at a greater rate than subordinate fish as a function of their dominance status (Buston, 2003; Fricke, 1979). Testing for differences in the body length change (Fig. 2C) over time between dominant and subordinate fish revealed a significant interaction ($F_{1,50} = 6.22$, $p = 0.02$, $\eta_p^2 = 0.07$), such that dominant fish grew longer (slope = 0.26) while subordinate fish did not (slope = 0.004). Testing for differences in body mass change (Fig. 2D) revealed a significant main effect of time ($F_{1,50} = 5.87$, $p = 0.02$, $\eta_p^2 = 0.11$), and the interaction term was not significant ($p = 0.14$). Thus, while both dominant and subordinate fish tended to grow to some degree, the dominant fish grew at a greater rate than subordinate fish and this effect was statistically better discerned in terms of body length change.

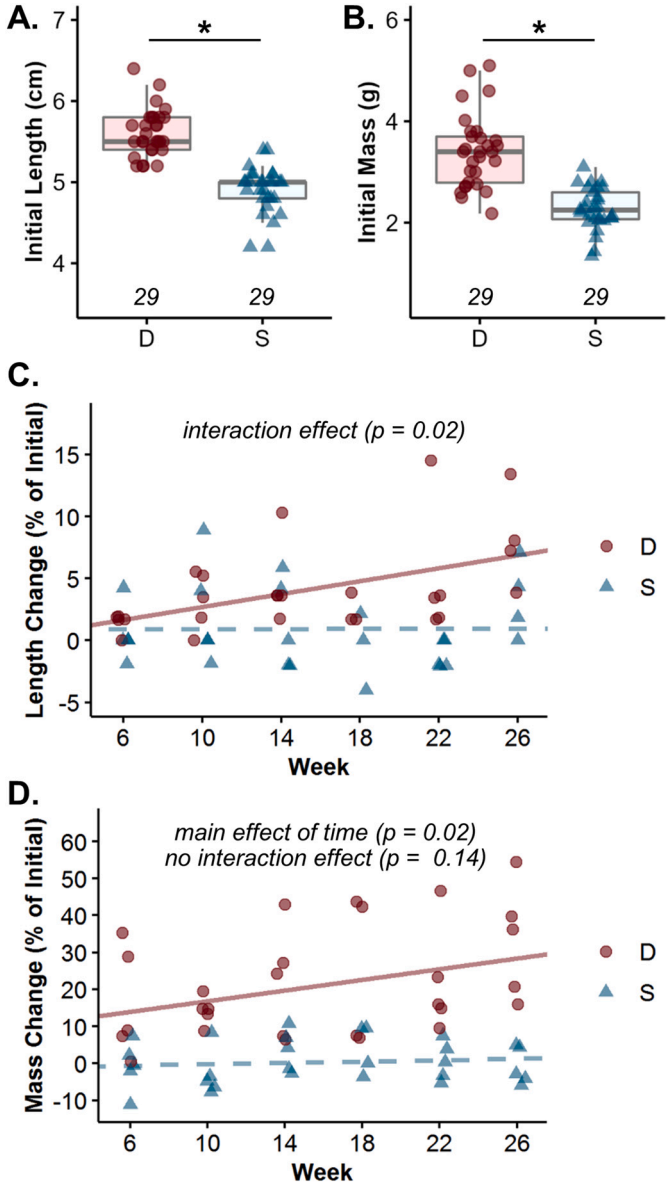

Fig. 2. The larger member of the pair became dominant and displayed increased body growth. Initial size of dominant and subordinate fish is shown in terms of A) body length (cm), and B) body mass (g). C) Difference in growth rate between dominant and subordinate fish was readily observed in body length change over time. D) Body mass change showed a similar trend as body length, though only the main effect of time was significant. The solid line indicates regression line for dominant fish, dashed line for subordinate fish. Asterisk indicates significant difference ($p < 0.05$). Numbers below the boxplots are number of individuals per group. Boxplots capture the inter-quartile range (IQR, first to third quartile), whiskers extend to the largest value within 1.5 IQR of the box, median is marked by a horizontal line within the boxplot.

3.2. Steroid hormones. Dominant sex-changing fish had E2 and 11KT between male and female levels, and greater 11KT than subordinate tankmates

Plasma E2 and 11KT were first assessed for differences in change over time between dominant sex-changing fish and their non-changing partners (Fig. 3A, C). Analysis of E2 revealed no effects of timepoint or status, indicating that E2 does not differentiate sex-changing fish from their non-changing tankmates and are stable during six months after the initiation of sex change. Analysis of 11KT, however, revealed a significant main effect of status ($F_{1,49} = 10.58$, $p = 0.003$, $\eta_p^2 = 0.12$), such that dominant sex-changing fish had greater 11KT than subordinate fish, and this difference was stable over time.

To compare hormone values of sex-changing fish to fish that had changed gonadal sex and to control males and females, dominant and subordinate fish were collapsed across timepoints and a one-way ANOVA was applied to both E2 and 11KT (Fig. 3B, D). Analysis of E2 revealed a significant effect of group ($F_{5,93} = 47.01$, $p < 0.001$, $\eta_p^2 = 0.71$). Post-hoc analysis showed that plasma E2 for dominant sex-changing fish was not different from their subordinate partners, nor was it different from that of new females or new males. Plasma E2 for all these groups was significantly different from both control males and control females (all $p < 0.01$), except for new males which were not significantly different from control males. Control males and females were significantly different from one another ($p < 0.001$). This suggests that plasma E2 rises above male-like levels in dominant sex-changing fish as well as in subordinate fish after pairing, but not to female-like levels. Analysis of 11KT also revealed a significant effect of group ($F_{5,85} = 42.96$, $p < 0.001$, $\eta_p^2 = 0.72$). 11KT was significantly different between all groups for every pairwise comparison (all $p < 0.05$) with two exceptions: new females and control females were not significantly different, and new males and control males were not significantly different. This suggests that dominant sex-changing fish have greater circulating 11KT than their subordinate tankmates until they develop vitellogenic oocytes, at which point their 11KT drops to a female-like level. And when 11KT drops in the new female fish, there is a corresponding elevation of circulating 11KT in the new male to a male-like level.

There was no significant difference in hormone ratio (E2:11KT) between dominant and subordinate fish over time (Fig. 3E). Collapsing across time and comparing all groups on hormone ratio (Fig. 3F) revealed a significant effect of group ($F_{5,84} = 79.09$, $p < 0.001$, $\eta_p^2 = 0.82$). All pairwise comparisons were significantly different (all $p < 0.01$) with two exceptions: dominant and subordinate fish were not significantly different, and new males and control males were not significantly different. Thus, in terms of E2:11KT ratio, dominant sex-changing fish fall between a male-like and a female-like profile alongside their subordinate tankmates. And while 11KT levels alone differentiate dominant and subordinate fish, the difference is not large enough to drive a significant difference in E2:11KT ratio.

3.3. Aggression assay. Dominant sex-changing fish have a male-oriented aggression bias, akin to control males and unlike control females

A number of fish displayed zero aggressive behaviors toward females or males. We observed zero aggression toward females in 11 of 27 (41 %) dominant fish, 21 of 27 (78 %) subordinate fish, 2 of 2 (100 %) new males, and 3 of 4 (75 %) control males. And we observed zero aggression toward males in 5 of 27 (19 %) dominant fish, 16 of 27 (59 %) subordinate fish, 1 of 2 (50 %) new males, and 2 of 4 (50 %) control females. These fish are included as zero values in the analyses below.

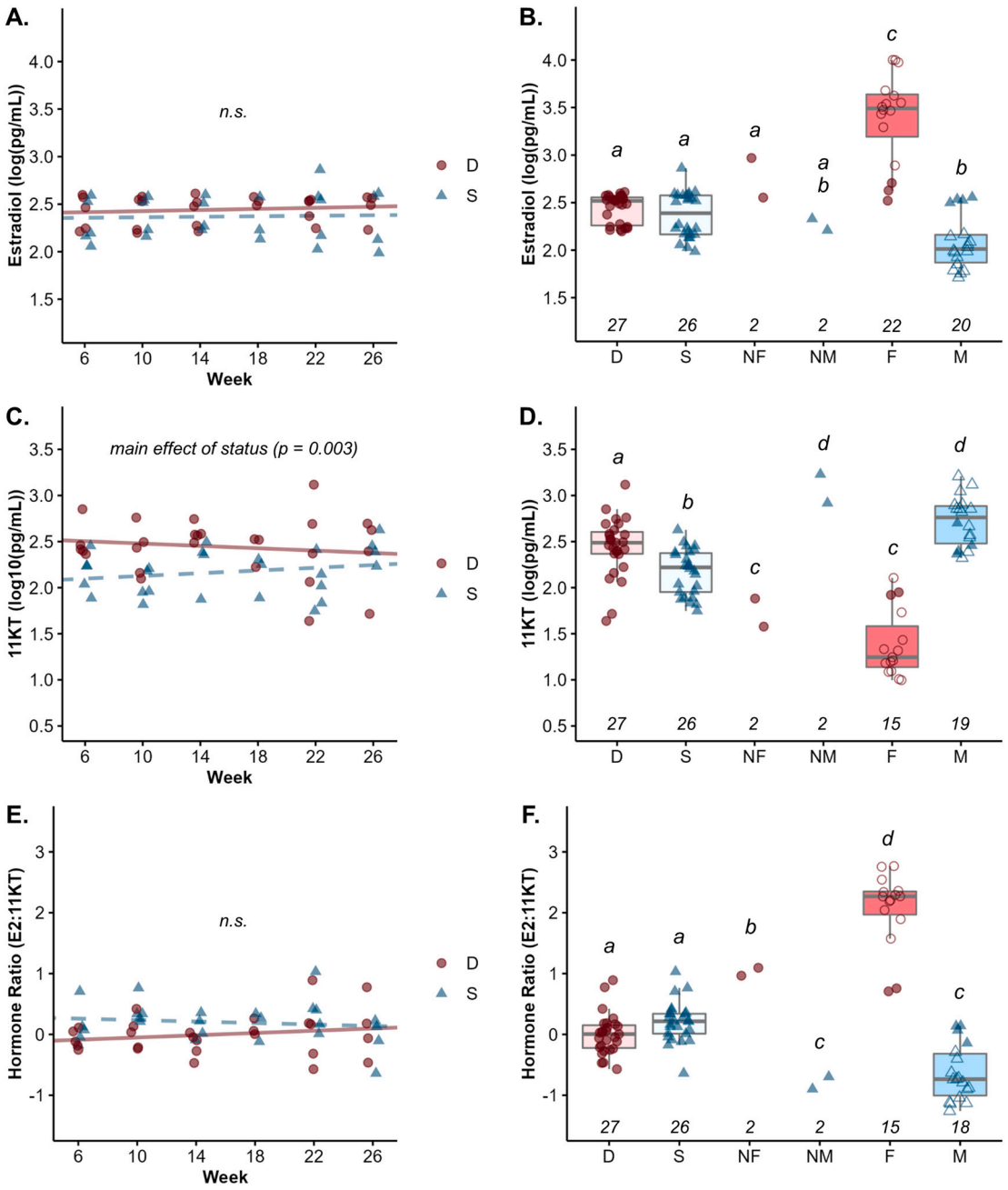

Fig. 3. Dominant sex-changing fish had E2 and 11KT between male and female levels, and greater 11KT than subordinate tankmates. A) Levels of E2 did not differ between dominant and subordinate fish over time. B) Comparing across all groups, dominants, subordinates, and new females displayed E2 between typical male and female levels, whereas new males were not different from control males. C) Levels of 11KT were higher in dominant than subordinate fish, and this difference was stable over time. D) While dominant and subordinate fish both had 11KT levels in between male and female levels, dominant fish had relatively higher 11KT than subordinates, and new females and new males fell in line with control females and males, respectively. E) The hormone ratio of E2:11KT did not differ between dominant and subordinate fish over time. F) Dominant sex-changing fish had an E2:11KT hormone ratio between control males and control females, and between new females and new males, alongside subordinate tankmates. For control males and female datapoints, solid shapes indicate fish collected in the present study and open shapes indicate previously published control fish (DeAngelis and Rhodes, 2016). Letters over boxplots distinguish groups that are significantly different from one another. Numbers below the boxplots are number of individuals per group. 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.

No differences were observed in female-oriented aggression between dominant and subordinate fish over time (Fig. 4A). Male-oriented aggression showed a significant main effect of time ($F_{1,50} = 4.77$, $p = 0.03$, $\eta_p^2 = 0.09$) but no effect of dominance status (Fig. 4C). This was due to a small increase in the overall prevalence of male-oriented aggression with time (slope = 0.03, in cube-root transformed units) that was not dependent on dominance status or sex change. However, there was no effect of time or status on F:M aggression ratio (Fig. 4E). Thus, while there was a tendency for all fish to be slightly more aggressive toward males at later timepoints, it was not a strong enough tendency to result in any significant effect on the F:M aggression bias.

Dominant and subordinate fish were then collapsed across time to compare them against control males and females as well as new females and new males on all measures of aggression (Fig. 4B, D, F). In these analyses there was a significant effect of group on female-oriented aggression ($F_{5,60} = 10.74$, $p < 0.001$, $\eta_p^2 = 0.47$), male-oriented

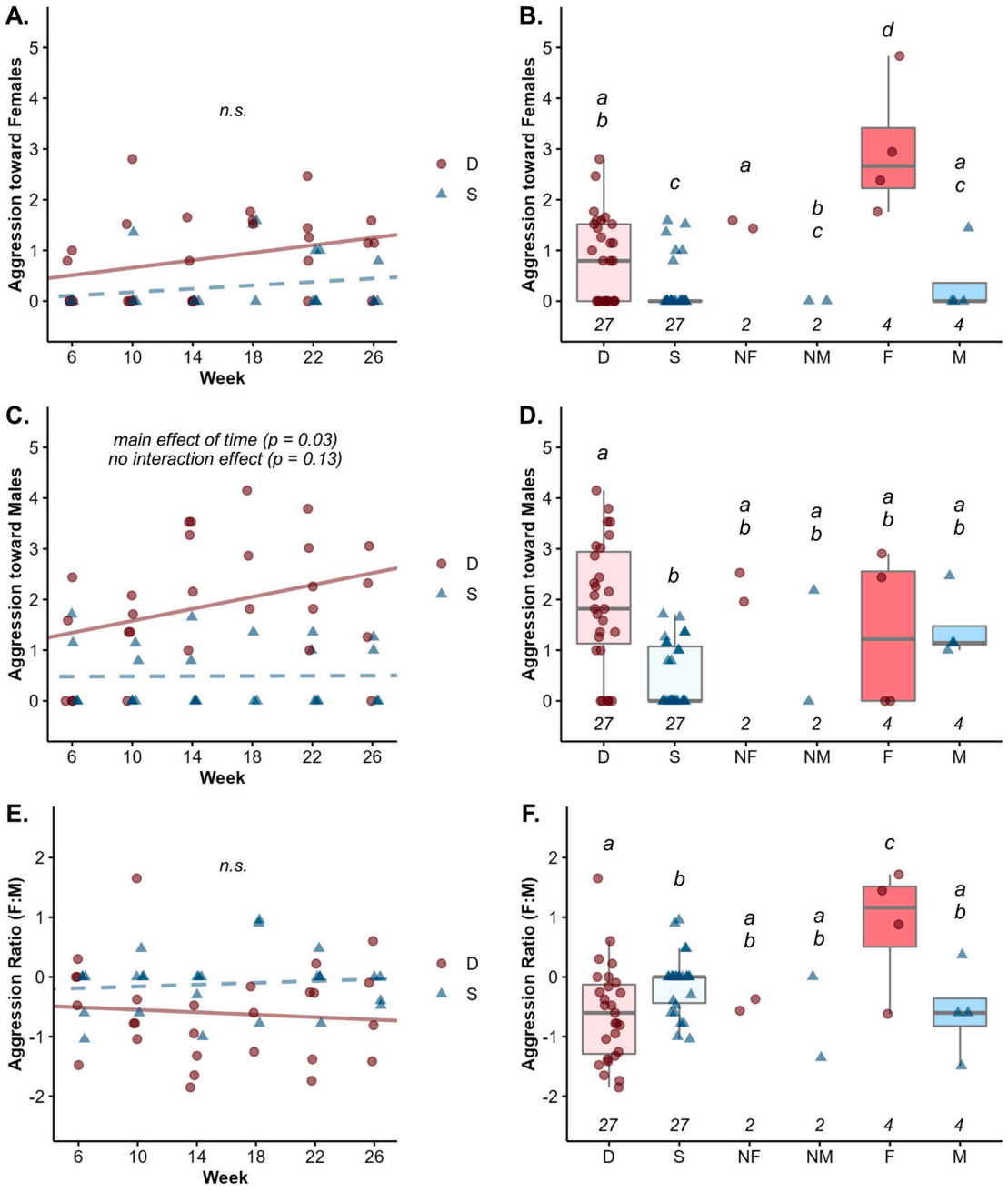

Fig. 4. Dominant sex-changing fish have a male-oriented aggression bias, akin to control males and unlike control females. A) Levels of female-oriented aggression did not differ between dominant and subordinate fish over time. B) Female-oriented aggression was significantly greater in control females than any other group, and dominant sex-changing fish displayed more female-oriented aggression than subordinate tankmates. C) Male-oriented aggression generally increased with time in both dominant and subordinate fish. D) Dominant sex-changing fish displayed significantly greater male-oriented aggression than subordinate tankmates but were otherwise comparable to all other groups. E) Aggression ratio (F:M) did not differ between dominant and subordinate fish over time. F) The aggression ratio (F:M) for control females was female-biased and significantly different from every other group. Dominant sex-changing fish were significantly more male-biased in their aggression compared to subordinate tankmates. Letters over boxplots distinguish groups that are significantly different from one another. Numbers below the boxplots are number of individuals per group. 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.

aggression ($F_{5,60} = 5.63$, $p < 0.001$, $\eta_p^2 = 0.32$), and F:M aggression ratio ($F_{5,60} = 3.88$, $p = 0.004$, $\eta_p^2 = 0.24$). In post-hoc analyses for all three of these measures, dominant sex-changing fish were significantly different from subordinate fish (all $p < 0.01$). Dominant fish displayed significantly greater aggression toward males and females than their subordinate tankmates, and they had a significantly more negative F:M aggression ratio indicating a stronger bias toward male-oriented aggression. Dominant fish were not significantly different from control males on any measure. However, dominant fish *were* significantly different from control females both in terms of female-oriented aggression and F:M aggression ratio (all $p < 0.001$). Altogether this indicates that the pattern of aggressive behavior displayed by dominant fish was much more male-like than female-like, with a strong bias toward male-oriented aggression. Interestingly, new females continued to display a pattern of aggression more like control males and dominant fish than control females, including a negative F:M aggression ratio reflecting a male-oriented aggression bias.

3.4. Parental care assay. Parental care behavior in dominant sex-changing fish is male-like, not female-like

Of all pairs of fish there was a single dominant-subordinate pair at the 22 week timepoint that displayed zero parental care behaviors. This pair was excluded from analysis. Testing for differences in the amount of parental care provided by dominant and subordinate fish over time (Fig. 5A) revealed a significant effect of status ($F_{1,48} = 5.44$, $p = 0.03$, $\eta_p^2 = 0.10$) such that dominant sex-changing fish provided greater parental care overall. A similar effect of status was found for the amount of time spent in the nest with eggs ($F_{1,48} = 4.29$, $p = 0.049$, $\eta_p^2 = 0.07$) (Fig. 5C). Thus by both measures dominant sex-changing fish displayed greater parental care than their subordinate partners, and this difference was stable over time.

Collapsing dominant and subordinate fish across timepoints and comparing them against control males and females as well as new females and new males revealed a significant effect of group on both the amount of parental care provided ($F_{1,88} = 13.84$, $p < 0.001$, $\eta_p^2 = 0.44$) as well as the amount of time spent in the nest with eggs ($F_{1,88} = 11.13$, $p < 0.001$, $\eta_p^2 = 0.39$) (Fig. 5B, D, respectively). For both measures, dominant fish displayed significantly greater parental care than control females (all $p < 0.01$) but did not differ significantly from control males. New females were also significantly greater than control females on both measures (all $p < 0.01$) but did not differ from control males. Surprisingly, the parental effort displayed by subordinate non-changing fish on both measures dropped significantly lower than control male levels (all $p < 0.01$), falling in line with what is typical for control females. But in new males the parental effort on both measures was back to a level typical of control males, and significantly greater than control females (all $p < 0.01$). Thus, the parenting behavior of dominant sex-changing fish is persistently male-like even until the point of gonadal sex change, while the parenting behavior of subordinate fish is suppressed below a male-like level until its partner has changed sex.

4. Discussion

The goal of this study was to evaluate the timecourse of behavioral sex change in protandrous anemonefish by comparing sex-changing fish directly to their subordinate partners and to control males and females. The results reveal a very different pattern of behavioral sex change in anemonefish as compared to protogynous or bidirectional sex changing fish. Relative to the rapid, gonad-independent pattern observed in protogynous and bidirectional species, behavioral sex change in anemonefish is not rapid and instead appears to occur after gonadal sex change, unlike all other sex changing fish that have been studied to this point. The timecourse for behavioral sex change in anemonefish supports a possible activational role of gonadal hormones, as the two fish in the study that changed gonadal sex did not yet have a fully female-like hormone profile and still behaved male-like. The patterns of aggression that we observed in sex-changing fish are consistent with field work

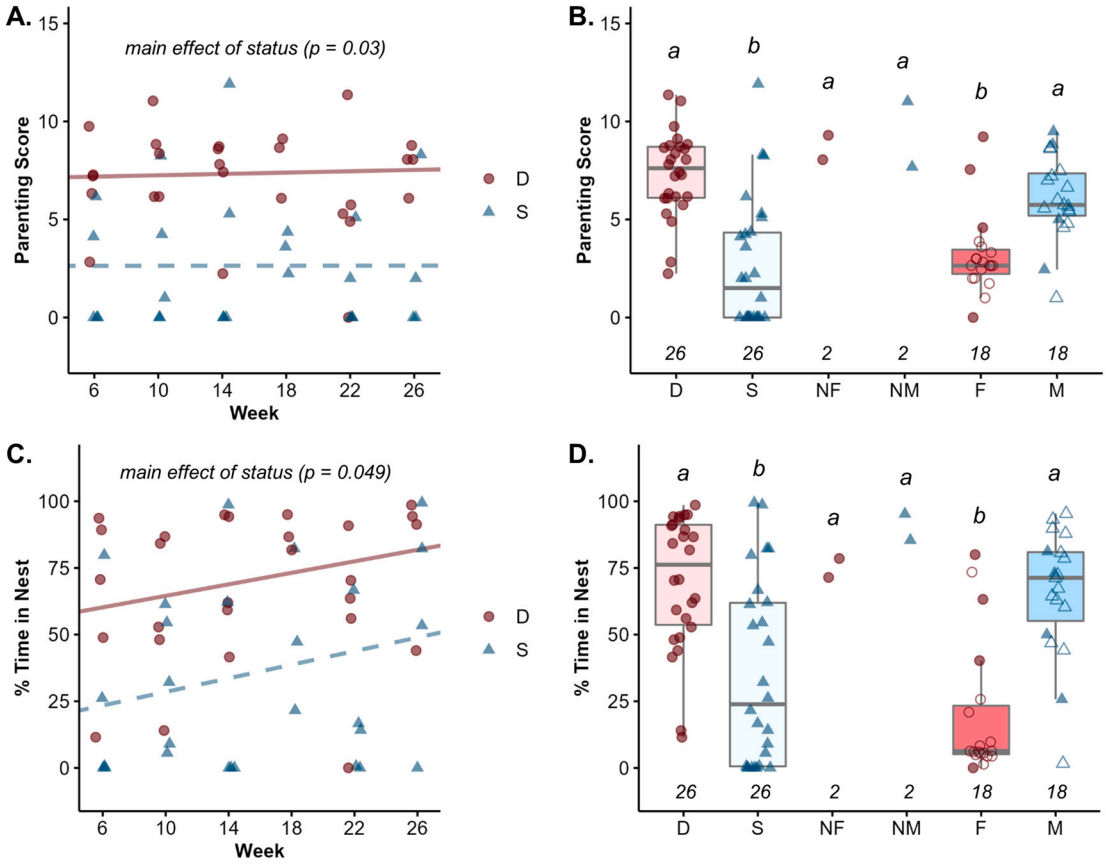

Fig. 5. Parental care behavior in dominant sex-changing fish is male-like, not female-like. A) Dominant sex-changing fish displayed a greater amount of parental care than subordinate fish, and this difference was stable over time. B) Dominant fish provided significantly more parental care than control females, comparable to control males as well as new females and new males. C) Time spent in nest followed the same pattern as the amount of parental care acts, with dominant fish spending more time in the nest than their subordinate tankmates and D) dominant fish spending significantly more time in the nest than control females, comparable to control males. Asterisk indicates significant difference ($p < 0.05$). For control males and female datapoints, solid shapes indicate fish collected in the present study and open shapes indicate previously published control fish (DeAngelis and Rhodes, 2016). Letters over data in panels B and D distinguish groups that are significantly different from one another. Numbers below the boxplots are number of individuals per group. 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.

showing elevated aggression in the anemonefish *A. melanopus* during sex change (Godwin, 1994a). While the studies differ on the basis of behavioral assay design and laboratory context, our results provide important new information that leads to an alternative interpretation of what elevated aggression during sex change may represent. It may not be indicative of female-like aggressive behavior, but rather of dominance ascension. Our results also corroborate past work describing a drop in 11KT below male-like levels and an increase in E2 above male-like levels with the onset of sex change in anemonefish (Godwin, 1994a; Godwin and Thomas, 1993). However, by assessing both dominant sex-changing fish as well as their subordinate non-changing partners, we come to the interpretation that this hormone profile is indicative of a state of reproductive stasis rather than gradual march toward a sex-reversed hormone profile. This is because both dominant and subordinate fish have a hormone profile that is intermediate to control males and females and similar to one another (Fig. 3E) (Dodd et al., 2019).

The timecourse of protandrous behavioral sex change in anemonefish is different from that in protogynous and bidirectional sex-changing species in three major ways. *First*, anemonefish do not change behavioral sex rapidly with the onset of sex change. This is in stark contrast to the well-established rapid behavioral sex change in protogynous and bidirectional species (Black et al., 2005a; Godwin et al., 1996; Grober and Sunobe, 1996; Hoffman et al., 1985; Nakashima et al., 2000; Reavis and Grober, 1999; Rodgers et al., 2007; Rodgers et al., 2005; Sakai et al., 2003; Sakai et al., 2002; Shapiro, 1981; Warner and Swearer, 1991). Anemonefish instead display a persistently male-like pattern of aggressive and parental behavior throughout six months after the initiation of sex change. *Second*, gonadal sex change is completed before behavioral sex change in anemonefish. New females, whose gonads contained vitellogenic oocytes and whose hormone profile had diverged from that of a sex-changing fish toward that of a control female, still displayed a male-like pattern of behavior. This pattern of behavioral sex change is notably unlike that observed in the bidirectional sex-changing Blue-banded goby which is rapid and precedes gonadal sex change in the protandrous and protogynous directions (Rodgers et al., 2007). The prolonged pattern in anemonefish where gonads change before behavior may be a feature of the unique life history and long lifespan of anemonefish (Buston and García, 2007; Sahm et al., 2019), or a general feature of unidirectional protandry, since once anemonefish or other protandrous species change sex to female that switch is permanent. *Third*, gonadal hormones and behaviors do not gradually move in the direction of the new sexual phenotype over the course of sex change. This is unlike protogynous sex change, in which behaviors associated with the new sex become gradually more frequent over the course of sex change and gonadal hormones gradually shift in the direction of the new sex (Godwin et al., 1996; Nakamura et al., 1989; Reavis and Grober, 1999). Instead of gradually becoming more hormonally or behaviorally "female-like", anemonefish appear to enter a discrete phase of life characterized by 1) a stable behavioral and hormonal phenotype distinct from that of either sex, 2) body growth, and 3) gradual accumulation of new cells in the anterior preoptic area (Dodd et al., 2019). It may be that gonadal sex change is not allowed to commence until sufficient change has occurred in the brain or sufficient body growth has occurred, then behavioral sex change would follow.

The behavioral profile of sex-changing anemonefish described here is unique and may be best characterized as a highly aggressive or dominant male phenotype. The behavior is male in the sense that the F:M aggression ratio is male-biased and parental care behavior is entirely male-like. However, the behavior is also unique in that sex-changing fish are fairly aggressive toward females as well as males, a feature uncharacteristic of typical male behavior. Past research that tested aggression in sex-changing anemonefish only tested female-oriented aggression, and found a similar pattern of elevated female-oriented aggression relative to control males (Godwin, 1994a). This was interpreted at the time as behavioral sex change. However, by expanding on this foundational past work and assessing both female- and male-oriented aggression in the present study, we found that the behavior of sex-changing fish is still ultimately more male-like, although unique in the display of aggression toward females and almost super-male in its overall magnitude. This unique pattern of behavior led us to consider the role of dominance status, leading to an alternative interpretation. Even in fish that do not change sex, ascent to dominance is associated with a rapid elevation in aggression (Alward et al., 2019; Maruska and Fernald, 2010), and higher rates of aggression are not only associated with dominance status across vertebrate species but are often essential to maintaining dominance (Holekamp and Strauss, 2016; Tibbetts et al., 2022). Our results support the notion that dominant sex-changing fish maintain a male-like direction to their behavior, but their unique social context of having newly established dominance and not yet gained enough in body size to make their dominance concrete (Buston, 2003; Fricke, 1979) leads them to express an exaggerated magnitude of aggression toward any opponent regardless of sex. For this reason, it is important to identify patterns of aggression that are truly diagnostic of sexual status per se when assessing behavioral sex change following dominance ascent. By this logic, the rapid emergence of new courtship and mating behavior in protogynous species may be considered better diagnostic markers for behavioral sex change than increased aggressive behavior alone (Black et al., 2005a; Godwin et al., 1996; Nakashima et al., 2000; Reavis and Grober, 1999; Rodgers et al., 2007; Rodgers et al., 2005; Warner and Swearer, 1991).

Using our novel aggression assay we found that sex-changing fish do not present a female-like sensitivity to the sex of the opponent until some time after they have become reproductively female. This ultimate change to a female-like pattern of aggression cannot be attributed to dominance status because sex-changing fish are dominant from the moment that sex-change is initiated through sex change and beyond into life as a female. Thus, the ultimate change to a female-like pattern of aggression is likely attributable to a change in sexual phenotype from male-like to female-like. The mechanistic basis of this shift in sexual behavioral phenotype should be unraveled in future work. The difficulty of disentangling dominance status from sexual status is a challenge and limitation inherent in this work as well as the study of socially-controlled sex-change generally. Still, the results of this study indicate that, in anemonefish, behavioral sex change does not occur until after gonadal sex change is complete, and that the elevated aggression displayed by sex-changing fish before gonadal sex change is a reflection of dominance status rather than a transition toward female-like behavior.

Given that E2 levels in the new females were not as high as reproductive females, activational effects of gonadal E2 could contribute to behavioral sex change after the hormones reach some threshold level. E2 treatment increases the frequency of aggressive behavior even in ambisexual (non-breeding) *A. ocellaris* (Iwata and Suzuki, 2020). Yet gonadal E2 alone cannot explain the aggression observed here in sex-changing fish as their subordinate tankmates had comparable levels of E2. There may also be a role for E2 synthesized locally in the brain, which has been implicated in regulating behavioral sex change and aggressive behavior in the protogynous Bluehead wrasse (Marsh-Hunkin et al., 2013; Prim et al., 2022). In anemonefish, brain aromatase expression is increased during sex change and is even further increased in females (Casas et al., 2016; DeAngelis et al., 2018), which is consistent with a possible role in mediating increased aggression. Nonapeptide signaling is also a likely mediator of sexually-differentiated aggression, as in anemonefish oxytocin and vasotocin cell number is differentiated by social status (Iwata et al., 2010a, 2010b), and oxytocin receptor expression is differentiated by sexual status (DeAngelis et al., 2018). Nonapeptide signaling has also been implicated in behavioral sex change, and aggressive behavior in particular, during protogynous sex change in a number of species (Black et al., 2004; Godwin et al., 2000; Grober and Sunobe, 1996; Lema et al., 2012; Semsar et al., 2001; Semsar and Godwin, 2004; Semsar and Godwin, 2003). Brain aromatase activity is a crucial regulator of social behavior across vertebrates, as are the nonapeptide signaling systems (Cornil, 2018; Cornil and Charlier, 2010;

Goodson and Thompson, 2010; Trainor et al., 2006). Research into behavioral sex change in anemonefish and other species provides a unique opportunity to study how brain aromatase and nonapeptide signaling are capable of regulating such dramatic behavioral plasticity against a shifting landscape of gonad-derived steroid hormones.

In order for sex change to work in monogamous species such as the anemonefishes, it is essential that the two members of the pair are co-ordinated in their reproductive and behavioral development. This is important so that when it comes time for the pair to reproduce in their new sexual roles for the first time, they are both ready to display the appropriate physiology and behavior. Beyond establishing a timecourse for behavioral and hormonal sex change, this study begat the remarkable observation that the new male partners of new female (sex-changed) fish appear to regulate their hormone secretion and behavior in coordination with the reproductive development of their new female partner. The subordinate fish in the pair must somehow sense the vitellogenic state of its newly sex-changed partner, and as a result regain a male-like hormone profile, gonadal function, and parental care behavior (i.e., become a "new male", as identified in this study) in preparation for the first reproductive act in their new sexual roles. No research to date has addressed this process or the possible mechanisms mediating it. Research into this process may provide a novel system for the study of how social stimuli (e.g. pheromones, visual cues, etc.) can exert dramatic control over an organism's reproductive and behavioral phenotype.

5. Conclusion

Rapid and gonad-independent behavioral sex change does not appear to be a universal characteristic of sex-changing fish species. In anemonefish, male-like aggressive and parenting behavior persists not only throughout the sex change period but even to the point when the gonads bear vitellogenic oocytes and the fish would be considered gonadally female. This may be the case as well for protandrous species more broadly, but further research is needed. With the timecourse and features of behavioral sex change now better described and candidate molecular mediators identified based on the broader literature, further research can better characterize the mechanisms underlying this unique pattern of social behavioral plasticity. These results also raise novel research questions about the coordinated reproductive development between the sex-changing fish and its partner.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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