Novel Behavioral Assays Reveal Sex Specific Behavioral Syndromes in Anemonefish

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Abstract

Sexually dimorphic behaviors are common across taxa, particularly in the contexts of parental care and territorial aggression. The false clown anemonefish *Amphiprion ocellaris* is unique among animals for its combination of female behavioral dominance and territoriality, protandrous sex change, and mutualistic symbiosis with sea anemones. Several laboratory studies have begun characterizing sex differences in parental care and aggression in this species, but aggression assays have mostly focused on intra-specific aggression where individual differences are large. The goals of this study were to expand the repertoire of behavioral assays available for *A. ocellaris*, establish repeatability of individual differences, identify assays that produce the most robust sex differences, and explore whether individual differences in correlated behaviors can be detected consistently across experimental contexts (i.e., whether behavioral syndromes can be detected). To this end, we measured 38 behaviors across 7 behavioral assays (parental care, large intruder, small intruder, male intruder, female intruder, reaction to a threat, nest maintenance) in 9 reproductively active *A. ocellaris* pairs under 3 different contexts (without eggs in the nest, with their own eggs, and with surrogate eggs). Behaviors were repeatedly measured three separate times (rounds) over repeated spawning cycles. We found 33 out of 38 behaviors were significantly individually repeatable across egg contexts and rounds, with an average intra-class correlation of 0.37. We found parental care, large intruder aggression, and female-oriented aggression assays produced the largest sex differences, where males performed 7-fold more egg care behaviors than females, while females performed 3.5-fold more aggressive behaviors toward a large interspecific intruder and 6.5-fold more bites toward a female intruder than males. Five different behavioral syndromes were observed in males but only one was observed in females. These results expand our understanding of sex differences in behavior and the division of labor in the iconic anemonefish. Future studies can use these assays to measure the behavioral sex of fish in the middle of sex change, in the study of behavioral plasticity, or in the study of the neuroendocrine bases of aggression and parental care in this unique species.

Keywords: Aggression, parental care, anemonefish, behavioral assay, sex difference, behavioral syndrome, sexual dimorphism, sex change, territoriality

Introduction

Behavioral sexual dimorphisms are common in sexually reproducing species. Species that care for their young and defend a territory often display a division of labor. For example, in many species (e.g., the African cichlid *Neolamprologous pulcher*, the marine goby *Microgobius gulosus*), males defend the territory while females care for eggs. The false clown anemonefish *Amphiprion ocellaris*, however, defies this norm. In this iconic coral reef fish, males are the primary caregivers, while females are responsible for territory defense. While paternal care is not uncommon in fish (e.g., in damselfishes, *Dascyllus reticulatus* and *Dascyllus aruanus,* and gobies *Pomatoschistus minutus* and *Paragobiodon echinocephalus*) (Gross & Sargent, 1985; Kuwamura et al., 1993; Lindström et al., 2006; Mizushima et al., 2000; Sakai et al., 2024), it is relatively unusual for females to take the primary role in territory defense (Kölliker, 2012). Even more unusual, anemonefish undergo protandrous (male-to-female) sex change (Fricke & Fricke, 1977; Liu et al., 2017). In the wild, *A. ocellaris* live in small, isolated groups comprised of one reproductively active α female, one reproductively active β male, and potentially several non-reproductive γ males (Fricke & Fricke, 1977). Should the α female be killed or removed, the β male takes her spot in the dominance hierarchy and undergoes protandrous sex change to become the new α female, while the most dominant γ male ascends to become the β male, and all subsequent fish move up one position in the dominance hierarchy (Fricke & Fricke, 1977). Thus, the same individual can display both male and female behaviors during their lifetime.

Anemonefish are also unique in that they are entirely dependent on their host sea anemone for survival (Da Silva & Nedosyko, 2016). Because of this dependence, they display extremely high levels of territorial defense (Laudet & Ravasi, 2022). Anemonefish will stand their ground and fight when faced with a significantly larger threat while most other coral reef fish species will swim away and hide in the rocks of the reef. As the largest and behaviorally dominant member of the group, females are the most aggressive (Iwata & Manbo, 2013). For example, female anemonefish have been known to attack much larger foes including human divers and sharks that stray too close to their host sea anemone (Iwata & Manbo, 2013; Laudet & Ravasi, 2022). This makes anemonefish ideal for studying female behavioral dominance and high levels of territorial aggression in females (Iwata & Manbo, 2013; Laudet & Ravasi, 2022; Rhoades & Blumstein, 2007).

Anemonefish's dependence on their sea anemone also means that they are particularly well suited to laboratory research. In the wild, anemonefish rarely stray more than a few meters away from the safety of their sea anemone, thus, they are comfortable in the confines of small (~75L) aquaria (Laudet & Ravasi, 2022). Additionally, in the lab, anemonefish readily reproduce and display normal parental care and nest behaviors even in the absence of a sea anemone (R. DeAngelis et al., 2017, 2020; Laudet & Ravasi, 2022; Phillips et al., 2020).

Recent laboratory studies have uncovered an unusual feature of anemonefish parental care behavior that allows for useful manipulations that are not possible for most other species. Anemonefish will care for eggs that are not their own and not kin with similar vigor as they would their own eggs (Phillips et al., 2020). Thus, parental care behavior can be evaluated by presenting fish with eggs from a different spawning pair and recording various parental care behaviors. This is a relatively unusual feature in fish as most fish will eat eggs that are not closely related (Pereira et al., 2017). The ability to use surrogate eggs can be useful for evaluating parental care behaviors in fish that may not have their own eggs (such as fish in the middle of sex change or in newly formed pairs) (Parker et al., 2022).

Recent laboratory studies have also begun to evaluate differences in levels of parental care and aggression displayed by male and female anemonefish (R. DeAngelis et al., 2020; Iwata & Manbo, 2013; Phillips et al., 2020). While males and females on average display different levels of parental care and territorial aggression, there is substantial individual variation that can obscure sex differences (R. S. DeAngelis & Rhodes, 2016; Iwata & Manbo, 2013; Parker et al., 2022; Phillips et al., 2020). Males also defend the nest and females also care for eggs, just to a lesser extent. The laboratory assays that have been used to establish differences in aggression in *A. ocellaris*, have mostly focused on intra-specific aggression toward an intruder male or female (Iwata & Manbo, 2013; Parker et al., 2022; Yaeger et al., 2014). In the assays used in Parker et al. (2020), males and females were removed from their home tank and paired with a novel male or female in a neutral territory. A sex difference was discerned using this assay, especially when considering the ratio of female-oriented to male-oriented aggression. Females tend to be more aggressive to females and less aggressive to males whereas males show the reverse pattern. However, there is substantial overlap in the distributions even for the ratio (Parker et al., 2022). Thus, additional work is needed to broaden the repertoire of behaviors to find the most robust, consistent assays that are demonstrative of the differential roles each sex has in the reproductive pair, especially for territorial aggression toward non-conspecifics. This is important not only for documenting the life history of an iconic species of fish but also useful for

mechanistic studies attempting to uncover neurological or neuroendocrine regulation of sex differences in parental care and territorial aggression (R. DeAngelis et al., 2018, 2020; R. S. DeAngelis & Rhodes, 2016; Iwata & Manbo, 2013; Laudet & Ravasi, 2022).

Additionally, while maintaining aquaria, we have noticed pronounced personalities among various anemonefish. When approaching an aquarium for basic maintenance, some females are passive and hide in the back of the tank, while others are extremely aggressive, and leap out of the water in an attempt to bite us. Moreover, some males display substantially more effort in caring for eggs than others (unpublished observations). However, to our knowledge, no one has evaluated the extent to which repeatable individual differences in aggressive and parental behaviors can be detected in the lab, i.e., whether laboratory housed anemonefish display behavioral syndromes, personalities, or reliable correlated sets of behaviors.

The goal of this study was twofold: First to find behavioral assays that most reliably differentiate the sexes and second to identify repeatable individual differences and examine if these individual differences constitute behavioral syndromes: correlated sets of behaviors that are repeatable across various environmental contexts (Bell et al., 2009).

Methods

Animals and Husbandry

Anemonefish used in this study were either first or second generation bred in-house from fish originally obtained from Ocean Reefs and Aquariums (Fort Pierce, FL), obtained as a gift from Peter Buston, or purchased from the pet trade. Fish were housed in 20-gallon tall (24” x 12” x 16”) or 25-gallon cube (18” ×18” ×18”) centrally filtered aquariums with a 6” terra cotta pot serving as a nest site and spawning substrate.

Domino damsels (*Dascyllus trimaculatus*) and hermit crabs (*Coenbita spp.*) used in this study were obtained from the pet trade and were housed together in three in 10-gallon (20” x 10” x 12”) aquariums consisting of three damselfish and one hermit crab. Filtration in was provided by sponge filters with reef rocks and terra cotta pots serving as cover.

In all tanks, salinity was kept at 1.026 specific gravity, temperature was kept between 26°-28°C, pH was kept between 8.0 and 8.4, and lighting was provided by fluorescent and LED lights over tanks on a 12:12 light schedule (lights on at 07:00 h and off at 19:00 h). Fish were fed twice daily with ReefNutrition TDO Chroma Boost pellets (Reed Mariculture Inc., Campbell, CA).

Ethical Note

None of the species used in this study are protected or endangered species. All experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Behavioral Assays

We utilized 7 behavioral assays to study the behaviors of 9 reproductively active pairs (n = 18, 9 males and 9 females). All assays were recorded between 15:00 and 17:00 to control for circadian confounds. For the purpose of this study, the term **focal animal** will signify the fish whose behavior was recorded, and the term **stimulus animal** will signify the animal introduced into the tank. After recording began, the researcher left the area to avoid influencing the results. We chose to use the same assay order across all conditions to keep order effects consistent in our analyses. Across all egg conditions and rounds, the order of behavioral assays were in the following order.

1. Parental Care

We utilized a variant of the parental care assay used in Phillips et al. (2020), Parker et al. (2022), R. DeAngelis et al., (2020), DeAngelis et al. (2018), and DeAngelis & Rhodes (2016). Males and females were recorded for 5 minutes with eggs in the nest, and for each individual, the following parameters were measured: nips, pectoral fans, tail fans, nest entrances, and time in the nest (See table 1 for descriptions of behaviors). In the no eggs condition, this assay was omitted.

2. Small Intruder Aggression

The day following the parental care assay, we tested the focal animals' reaction to a small intruder near the nest. To test this, a hermit crab was placed near the nest. To control for movement around the tank, we fashioned a 'leash' out of a centrifuge tube glued to the crab's shell with a small stretch of fishing line connecting it to another centrifuge tube that was placed under the terra cotta pot. This prevented the crab from going more than a certain radius (~ 10 cm.) away from the nest. Focal animal's behaviors were recorded for 10 minutes, and for each individual, we measured latency, bites, and strikes.

3. Large Intruder Aggression

The day after the small intruder assay, we tested the focal animals' reaction to a large intruder in the tank. A domino damselfish (*Dascyllus trimaculatus*) was used for this assay due to it being a natural competitor with *A. ocellaris* (Hayashi et al., 2020). This assay was modified from the assay used in DeAngelis et al. (2020). In this study, we only used one damsel as opposed to the three used in the cited study to allow for more targeted aggression. The stimulus animal was placed into the tank for 10 minutes and the following behaviors were recorded: latency, bites, strikes, displays, and chases/charges.

4. Male-Oriented Aggression

The day following the large intruder assay, we tested the focal animals' reaction to a conspecific male. We chose males that had previously spawned and recorded the stimulus animal's size relative to the focal male to test if there was an effect of relative size on aggression. This assay along with the female-oriented aggression assay were modified from the assays used in Parker et al. (2022) in two relevant ways. First, in this study, the focal pair was kept together and second, the assay took place in their home tank as opposed to a novel tank. Due to high levels of aggression from some fish, the male was only placed in the tank for 2 minutes as any longer was deemed to be a risk to the stimulus animal's health. The following behaviors were recorded: latency, bites, strikes, displays, and chases/charges.

5. Female-Oriented Aggression

The day after the male-oriented aggression assay, we tested the focal animals' reaction to a conspecific female. Only females that had previously spawned were used, and we recorded the stimulus animal's relative size to the focal female to test if there was an effect of relative size on aggression. For similar reasons as the male-oriented aggression assay, the stimulus animal was only placed in the tank for 2 minutes. The following behaviors were recorded: latency, bites, strikes, displays, chases/charges.

6. Immediate Reaction to a Threat

The day after the female-oriented aggression assay, we tested the focal animals' immediate reaction to a large threat, a proxy for boldness. To test this, a researcher placed their hand in the aquarium and behaviors were recorded for 10 seconds. The following behaviors were recorded: latency, bites, strikes, displays, and chases/charges.

7. Nest Maintenance

The same day as the immediate reaction to a threat assay, we tested how the focal animals would respond to a nest contaminant. To test this, we placed a small lava rock (~ 4 cm.) in the back of the terra cotta pot and recorded the following behaviors for 5 minutes: latency, bites, and strikes. On a few occasions, the pair would successfully remove the rock from the pot prior to the end of the 5 minutes. In this case, the assay was redone the following day.

Egg Conditions

To determine how the various behaviors measured in the behavioral assays were impacted by the presence of eggs, we repeated all of the behavioral assays across three egg conditions. Assays were recorded when the sample pair had their own eggs (Own), surrogate eggs of a similar age from a different pair (Surrogate), and no eggs (None / No). In the no eggs condition, the parental care assay was omitted. To control for hormonal changes across the breeding cycle, surrogate eggs were exchanged with the pair's own eggs the day after spawning such that the pair would be in the same position in its breeding cycle in both conditions (R. S. DeAngelis & Rhodes, 2016). When exchanging surrogate eggs for the pair's own eggs, 24h was allowed for acclimation to the novel terra cotta pot (Unpublished observation, anemonefish require 24h of acclimation to behave normally in the surrogate condition and for behaviors to stabilize). The no egg condition began the day after all eggs had naturally hatched or died, and if the pair spawned again before all assays could be completed, the assays were completed in the next inter-spawn period.

Rounds

To measure the repeatability of the behavioral assays, we repeated all of the assays in each egg condition three times.

Behavioral Analysis

All assays were recorded using Samsung HMX-W300 cameras. 24 Hours prior to the first assay until the end of the last behavioral assay, the camera and tripod were set up in front of the tank to acclimatize the pairs to recording. BORIS (v. 8.22) was used to analyze each assay (Friard & Gamba, 2016). The timer for each analysis began after the researcher's hand left the tank or the introduced fish was added and ended after the allotted amount of time had passed. Behaviors were only recorded in this time window.

Parental Behaviors

Time in nest	More than half of the focal animal's body is inside the terra cotta pot.
Entrance	The number of times the focal animal enters the terra cotta pot.
Pectoral fan	The focal animal waves its pectoral fins at the eggs to oxygenate them.
Tail fan	The focal animal 'wiggles' on the eggs to oxygenate them.
Nip	The focal animal mouths/ bites at the eggs.

Table 1A. Descriptions of parental behaviors

Aggressive Behaviors

Latency	The time from the start of the video to the first behavior. If no behavior was observed, the length of the assay was counted as the latency.
Chase/Charge	The focal animal swims quickly at the stimulus animal.
Display	The focal animal shows its side to the stimulus animal. This may include floating at an angle, tilting its body, and flexing its body.
Strike	The focal animal bats the stimulus animal with its tail or rams the stimulus animal with its head.
Bite	The focal animal bites the stimulus animal.

Table 1B. Descriptions of aggressive behaviors

The terms **sum of parental behaviors, sum of aggressive behaviors,** or **sum of behaviors** indicate the sum of measured behaviors in each assay not including latency. In the parental care assay, nest entrances and time in nest do not contribute toward this sum.

Aggression Ratio

As in Parker et al. (2022), a female to male aggression ratio was calculated. The sum of aggressive behaviors in the female-oriented aggression assay was divided by the sum of aggressive behaviors in the male-oriented aggression. We added a value of 1 to both of these values to avoid 0s in the denominator. The female/male aggression ratio was then log10 transformed due to non-normal distribution. This results in a measure centered around 0 where positive values indicate a female aggression bias, and negative values indicate a male aggression bias.

Statistical Analyses

R (4.2.3) was used for statistical analyses with a $P<0.05$ considered statistically significant (R Core Team, 2024).

Linear Regression

The lmer() function from the package "lme4" was used to fit linear mixed-effects models to the behaviors in each assay. We modeled both the sum of behaviors recorded in the assay and the count of each individual behavior within each assay. Fixed effects included round, egg condition and sex and all interactions. Note that round and egg condition are within-subjects fixed effects whereas sex is between-subjects. The fish id was entered as a random effect to account for repeated measures. In instances where residuals were not normally distributed, we transformed data with an appropriate transformation (see Supplementary Excel Files). In cases where we log transformed data, we added 1 to all values to avoid 0s. To extract P-values for individual factors with multiple levels (e.g., round, egg condition), we used the anova() function with the test = "Chisq" option specified. Pairwise comparisons were computed using the emmeans() functions from the package "emmeans" with the "tukey" P-value adjustment specified. Partial eta squared (η^2_p) for mixed effects models and eta squared (η^2) for linear regression models were calculated using the eta_squared() function from the package "effectsize".

Repeatability

To calculate repeatability for each model, we divided the variance of the individual by the sum of residual variance + individual variance. This produces the intra-class correlation (ICC) of the corrected values. To calculate the significance of repeatability, we extracted the significance of the random effect using the function ranova() from the package 'lmerTest'.

Principal Component Analysis

The princomp() function was used to analyze principal components based on each of the average of the sum of behaviors collapsed across all egg conditions and rounds. The cor = TRUE option was specified to scale the data which was necessary control for the various timeframes assays were conducted in (10s – 5m). Normality of principle components (PCs) were assessed using the Kolmogorov-Smirnov test, all data were normally distributed. To assess for significant sex differences in our PCA analyses, we used two-sided t-tests to compare males and females. To assess the variation within a principal component, we used linear regression models to fit principal component by sex, and extracted the R^2.

Results

Parental Care: Males contribute significantly more time and effort toward parental care than females.

We began our experiment by utilizing an assay that is well known to produce large and significant differences between males and females: a parental care assay (R. DeAngelis et al., 2017, 2018; R. S. DeAngelis & Rhodes, 2016; Parker et al., 2022; Phillips et al., 2020).

Unsurprisingly, we replicated the finding that the sum of parental behaviors was highly sexually dimorphic (Square root transformed, $F_{1,16} = 26.536$, $P<0.001$, $\eta^2_p = 0.73$; Fig. 1 A), as well as time spent in the nest (No transformation, $F_{1,16} = 25.966$, $p<0.001$, $\eta^2_p = 0.62$; Fig. 1 B). Males spent over 3 times as much time in the nest and over 7 times as much effort on parental care compared to females. As in Phillips et al., (2020), we found that pairs behaved similarly regardless of whether they were caring for their own eggs or surrogate eggs (All $P>0.05$). In addition, we found that the number of the eggs in the nest had a significant positive effect on the sum of parental behaviors (Square root transformed, $F_{1,56} = 6.076$, $P = 0.016$, $\eta^2_p = 0.07$), but not on time spent in the nest ($P = 0.105$; see Supplementary Excel File 1 for ANOVA tables, and 2 for means and standard errors).

In addition to parental care behaviors and time in the nest, we also measured the number of nest entrances. Males entered the nest significantly more than females (Square root transformed, $F_{1,17} = 25.933$, $P<0.001$, $\eta^2_p = 0.39$; Fig. 1 C). Further, the relationship between time in the nest and number of nest entrances was opposite for males as compared to females. This was indicated by a significant interaction between sex and time spent in the nest ($F_{1,54} = 27.193$, $P<0.001$, $\eta^2_p = 0.34$); males spent less time in the nest as nest entrances increased, while females spent more time in the nest as nest entrances increased (Fig. 1 D). This reverse pattern is likely a reflection of the division of labor regarding parental care and territorial defense. Males spend the majority of their time caring for eggs and only rarely leave the nest to defend the territory or explore the environment, while females spend the majority of their time out of the nest exploring the environment. Thus, in males, an increase in nest entrances indicates increased time out of the nest, while in females, an increase in nest entrances indicates increased time in the nest (see Supplementary Excel File 1 for ANOVA tables, 2 for means and standard errors, and 3 for pairwise comparisons).

We found that all of the recorded parental care behaviors were significantly repeatable (see table 2 for ICC and P-values).

Modeled Behavior	Model ICC	P-Value of Random Effect
Sum of Parental Behaviors	0.374	**<0.001**
Nips	0.416	**<0.001**
Pectoral Fans	0.321	**<0.001**
Tail Fans	0.295	**<0.001**
Time in the Nest	0.649	**<0.001**
Nest Entrances	0.280	**0.002**

Table 2. Model ICC and P-value of the random effect for all modeled behaviors in the parental care assay.

Small Intruder Aggression: Pairs are more aggressive with eggs.

For the small intruder aggression assay, we reasoned that a hermit crab could be used to model the fish's response to a small intruder.

We found no significant sex differences in any of the recorded behaviors in the small intruder aggression assay (All $P>0.05$). Interestingly however, we found a significant effect of the egg condition on the sum of aggressive behaviors (No transformation, $F_{2,118} = 3.284$, $P = 0.041$, $\eta^2_p = 0.05$). Pairs were slightly more aggressive with eggs in the nest (own or surrogate) compared to with no eggs in the nest though these effects were not significant in post-hoc tests (see Supplementary Excel File 3 for pairwise comparisons, 4 for ANOVA tables, and 5 for means and standard errors).

We also found that bites significantly increased as rounds progressed, particularly between rounds 1 and 2 (Square root transformed, $F_{2,119} = 3.815$, $P = 0.035$, $\eta^2_p = 0.06$; Fig. 2 A; see Supplementary Excel File 3 for pairwise comparisons,4 for ANOVA tables, and 5 for means and standard errors).

All of the recorded behaviors were significantly repeatable (see table 3 for ICC and P-values).

Modeled Behavior	Model ICC	P-Value of Random Effect
Sum of Aggressive Behaviors	0.657	**<0.001**
Latency	0.436	**<0.001**
Strikes	0.661	**<0.001**
Bites	0.461	**<0.001**

Table 3. Model ICC and P-value of the random effect for all modeled behaviors in the small intruder aggression assay.

Large Intruder Aggression: Females contribute more toward nest defense.

To model the fish's response to a large intruder, we introduced a domino damselfish *(Dascyllus trimaculatus*), a fish that is known to inhabit similar areas of the reef as *A. ocellaris,* and is known to be a threat to *A. ocellaris* eggs (R. DeAngelis et al., 2020; Hayashi et al., 2020). A previous study in our lab used a similar assay, however in that assay, 3 damselfish were introduced into the focal pair's tank, which may have acted to dilute aggression across several individuals (R. DeAngelis et al., 2020). Thus, in this assay, we only used a single damselfish to better model the focal animals' aggression toward a large non-conspecific intruder.

We found a significant effect of sex on the sum of aggressive behaviors (No transformation, $F_{1,16}=10.548$, $P = 0.005$, $\eta^2_p = 0.40$; Fig. 2 B), and the number of displays (Square root transformation, $F_{1,16}=15.150$, $P=0.001$, $\eta^2_p = 0.49$). Females produced more than 3.5 times the number of aggressive behaviors than males including over double the number of displays (see Supplementary Excel File 6 for ANOVA tables, and 7 for means and standard errors).

Unlike in small intruder aggression, we did not find any significant effect of egg condition on any of the modeled behaviors (All $P>0.05$)

We did however find a significant effect of round on the sum of aggressive behaviors (No transformation, $F_{2,128} = 21.935$, $P<0.001$, $\eta^2_p = 0.26$; Fig. 2 C), chases and charges (Cube root transformation, $F_{2,128} = 11.122$, $P<0.001$, $\eta^2_p = 0.15$), displays, (Square root transformation, $F_{1,128}=16.210$, $P<0.001$, $\eta^2_p = 0.20$), and latency (Log transformation, $F_{2,128} = 9.177$, $P= 0.002$, $\eta^2_p = 0.013$; Fig. 2 D). Pairs increased in aggression as rounds progressed as indicated by a

2-fold increase in the number of aggressive behaviors including a 2.5-fold increase in chases/charges, a 1.8-fold increase in displays, and a 77% reduction in latency between round 1 and round 3 (see Supplementary Excel File 3 for pairwise comparisons, 6 for ANOVA tables, and 7 for means and standard errors).

We found that all of the recorded behaviors were significantly repeatable (see table x for ICC and P-values).

Modeled Behavior	Model ICC	P-Value of Random Effect
Sum of Aggressive Behaviors	0.592	**<0.001**
Latency	0.192	**<0.001**
Displays	0.228	**<0.001**
Chases/Charges	0.798	**<0.001**
Strikes	0.415	**<0.001**
Bites	0.174	**0.001**

Table 4. Model ICC and P-value of the random effect for all modeled behaviors in the large intruder aggression assay.

<u>Male-Oriented Aggression: Pairs get more aggressive to males over time.</u>

To model conspecific aggression, we utilized the two conspecific aggression assays and aggression ratio used in Parker et al. (2022) (male and female-oriented aggression) with some relevant modifications. First, we kept the focal pair together in their home tank as opposed to separating them and putting them in a novel tank, and second, we shortened the length of the assay from 15 minutes to 2 minutes as preliminary studies found that pairs were much more aggressive toward the stimulus animal when introduced to their home territory (Parker et al., 2022, unpublished observation).

As in Parker et al. (2022), we did not find a significant difference between males and females for any of the measured behaviors in the male-oriented aggression assay (All $P > 0.05$).

However, we did find a significant effect of egg condition on the sum of aggressive behaviors (Log transformed, $F_{2,128} = 5.940$, $P = 0.003$, $\eta^2_p = 0.08$) displays (No transformation, $F_{2,128} = 3.338$, $P < 0.039$, $\eta^2_p = 0.05$) and latency (Log transformed, $F_{2,128} = 7.153$, $P = 0.001$, $\eta^2_p = 0.10$; Fig. 3 A). Pairs displayed more aggressive acts with no eggs in the nest compared to in the own egg condition as indicated by a 1.3-fold increase in the sum of aggressive behaviors and displays and a 66% decrease in latency. Interestingly, no differences between the no eggs and surrogate eggs conditions were observed (see Supplementary Excel File 3 for pairwise comparisons, 8 for ANOVA tables, and 9 for means and standard errors).

Similar to the large intruder aggression assay, we found a significant effect of round on the sum of aggressive behaviors (Log transformed, $F_{2,128} = 10.195$, $P<0.001$, $\eta^2_p = 0.14$), latency (Log transformed, $F_{2,128} = 17.592$, $P<0.001$, $\eta^2_p = 0.22$; Fig. 3 B) and displays (No transformation, $F_{2,128} = 9.995$, $P<0.001$, $\eta^2_p = 0.14$), where pairs produced 1.2 times more aggressive behaviors, 1.5 times more displays, and reduced latency over 76% to address the stimulus animal between round 1 and round 3. We also found a significant interaction between eggs and round for displays (No transformation, $F_{4,128} = 2.495$, $P = 0.046$, $\eta^2_p = 0.087$). This was caused by slightly fewer displays in the own egg condition relative to the other conditions in round 2 that did not occur in subsequent rounds (see Supplementary Excel File 3 for pairwise comparisons, 8 for ANOVA tables, and 9 for means and standard errors).

Interestingly, we found that all of the recorded behaviors were significantly repeatable except for latency (see table 5 for ICC and P-values).

Modeled Behavior	Model ICC	P-Value of Random Effect
Sum of Aggressive Behaviors	0.156	**0.003**
Latency	0.094	0.06
Displays	0.339	**<0.001**
Chases/Charges	0.263	**<0.001**
Strikes	0.173	**0.001**
Bites	0.300	**0.001**

Table 5. Model ICC and P-value of the random effect for all modeled behaviors in the male-oriented aggression assay.

Female-Oriented Aggression: Females preferred direct aggression while males preferred indirect aggression.

In female-oriented aggression, we found that in several instances, the size of the stimulus female relative to the focal female had a significant effect in our models. Thus, in all analyses of behaviors in female-oriented aggression, we added a term 'Relative Size' with levels "Smaller", 'Similar", or "Larger" to our models with no interactions.

While we did not find a significant effect of sex on the sum of aggressive behaviors (P>0.05), sex differences emerged when we analyzed the behaviors separately. We observed a significant effect of sex on the number of displays (No transformation, $F_{1,16}$ = 9.250, P= 0.008, η^2_p = 0.37; Fig. 3 C) and bites (Square root transformed, $F_{1,13}$ = 15.158, P=0.002, η^2_p = 0.54; Fig. 3 D). Males produced nearly double the number of displays than females, while females bit the stimulus animal over 6.5 times more than males. Taken together, these data suggest males preferred indirect aggression (displays), while females preferred direct aggression (bites) (see Supplementary Excel File 10 for ANOVA tables, and 11 for means and standard errors).

The size of the stimulus female relative to the focal female also had a significant effect on female-oriented aggression. This was apparent for bites (Square root transformed, $F_{2,138}$ = 7.922, P<0.001, η^2_p = 0.10), and chases/charges ($F_{2,138}$ = 5.259, P = 0.006, η^2_p = 0.07; Fig. 3 E). Pairs chased/charged at smaller stimulus females over 3 times as much as larger stimulus females, but bit similar or larger sized stimulus females slightly more than smaller stimulus females. Pairs did not significantly discriminate between larger and similarly sized stimulus animals (see Supplementary Excel File 3 for pairwise comparisons, 10 for ANOVA tables, and 11 for means and standard errors).

We also found a significant effect of egg condition on the sum of aggressive behaviors (No transformation, $F_{2,127}$ = 7.960, P<0.001, η^2_p = 0.11), chases and charges (Square root transformed, $F_{2,128}$ = 7.044, P= 0.001, η^2_p = 0.10), strikes (No transformation, $F_{2,127}$ = 3.915, P = 0.022, η^2_p = 0.06), and latency (Log transformed, $F_{2,128}$ = 3.551, P=0.031, η^2_p = 0.05). Similar to male-oriented aggression, pairs addressed the stimulus more rapidly in the none or surrogate conditions, and the number of aggressive acts was higher relative to the own eggs condition. When comparing the none condition to the own condition, pairs addressed the stimulus females 62% faster, and produced 1.4-times the number of aggressive behaviors in the none condition relative to the own condition including a 2.8-fold increase in chases/charges, and a 1.4-fold increase in strikes. Similar to male-oriented aggression, there was no significant difference in aggression in the none condition relative to the surrogate condition except in the case of chases/charges, where pairs were more aggressive with no eggs in the nest. There was also a significant interaction between egg condition and sex (No transformation, $F_{2,126}$ = 3.957, P = 0.021, η^2_p = 0.06). Females were significantly less aggressive in the own egg condition compared to the no egg condition and surrogate egg conditions, while males showed no significant difference in aggression based on egg condition (see Supplementary Excel File 3 for pairwise comparisons, 10 for ANOVA tables, and 11 for means and standard errors).

Similar to the large intruder aggression and male-oriented aggression assays, we again found a significant effect of round on the sum of aggressive behaviors (No transformation, $F_{2,126}$ = 5.831, P = 0.004, η^2_p = 0.08), displays (No transformation, $F_{2,126}$ = 8.138, P<0.001, η^2_p = 0.11), and latency (Log transformed, $F_{2,127}$ = 16.046, P<0.001, η^2_p = 0.20). Pairs got more aggressive and more rapidly addressed the stimulus female as rounds progressed, resulting in a 1.4-fold increase in aggressive behaviors including a 1.6-fold increase in displays, as well as a 60% decrease in latency to address the stimulus female between round 1 and round 3 (see Supplementary Excel File 3 for pairwise comparisons, 10 for ANOVA tables, and 11 for means and standard errors).

Similar to in male-oriented aggression, we found all modeled behaviors were significantly repeatable except latency (see table 6 for ICC and P-values).

Modeled Behavior	Model ICC	P-Value of Random Effect
Sum of Aggressive Behaviors	0.452	**<0.001**
Latency	0.068	0.153
Displays	0.255	**<0.001**
Chases/Charges	0.260	**<0.001**
Strikes	0.279	**<0.001**
Bites	0.311	**<0.011**

Table 6. Model ICC and P-value of the random effect for all modeled behaviors in the female-oriented aggression assay.

Aggression Ratio: Females and Males are more aggressive toward their own sex.

We replicated the finding in (Parker et al., 2022) that there was a significant sex difference in aggression ratio (Log10 transformed, $F_{1,16}$= 10.332, P= 0.005, η^2_p = 0.39); females were more aggressive toward females, while males were more aggressive toward males (Fig. 3 F). Further, we found no significant effect of egg condition, round, or any of the interactions present in the model (P >0.05) (see Supplementary Excel File 12 for the ANOVA table, and 13 for means and standard errors)

The female-male aggression ratio was not significantly repeatable (Log transformed, Model ICC: 0.059, Random effect P = 0.208) suggesting excessive within individual variation when measuring behaviors recorded across two assays.

Immediate Reaction to a Threat: Females are significantly bolder than males.

To measure boldness, we measured the focal animal's response to a large threat (a researcher's hand) within a short timeframe (10s).

We found a significant effect of sex on the sum of aggressive behaviors (No transformation, $F_{1,16}$ = 4.620, P = 0.047, η^2_p = 0.22; Fig. 4 A), chases and charges (Log transformed, $F_{1,16}$ = 5.172, P = 0.037, η^2_p = 0.24), and latency (No transformation, $F_{1,16}$ =8.618, P = 0.010, η^2_p = 0.35; Fig. 4 B). Females produced 1.8 times the number of aggressive behaviors including being the only sex that was observed producing chases/charges, and addressed the threat nearly 60% faster than males (see Supplementary Excel File 14 for ANOVA tables, and 15 for means and standard errors).

We did not find any significant effect of egg condition on any of the modeled behaviors (All P >0.05). While there was no significant main effect of egg condition for displays, we did find a significant interaction between egg condition and sex for displays (No transformation, $F_{2,128}$ = 3.130, P = 0.047, η^2_p = 0.05) and latency (No transformation, $F_{2,128}$ = 5.226, P= 0.007, η^2_p = 0.07). Post-hoc tests indicated that females were slightly more aggressive with eggs in the nest, while males were slightly less aggressive with eggs in the nest, showing the division of labor present in anemonefish pairs (see Supplementary Excel File 3 for pairwise comparisons, 14 for ANOVA tables, and 15 for means and standard errors).

Again, we found a significant effect of round on latency (No transformation, $F_{2,128}$ = 3.306, P = 0.040, η^2_p = 0.06), pairs addressed the threat 33% faster in round 3 relative to round 1 (see Supplementary Excel File 3 for pairwise comparisons, 14 for ANOVA tables, and 15 for means and standard errors).

We found that all of the recorded behaviors were significantly repeatable except for strikes and bites (see table 7 for ICC and P-values). This is likely caused by the rarity of strikes and bites recorded in this assay. Across the 81 videos analyzed, there was a total of 8 bites and 8 strikes recorded, with 7 of the bites and 7 of the strikes produced by 3 females (C11F, C15F, and T18F).

Modeled Behavior	Model ICC	P-Value of Random Effect
Sum of Aggressive Behaviors	0.468	**<0.001**
Latency	0.328	**<0.001**
Displays	0.382	**<0.001**
Chases/Charges	0.242	**<0.001**
Strikes	0.087	0.073
Bites	0.74	0.123

Table 7. Model ICC and P-value of the random effect for all modeled behaviors in the immediate reaction to a threat assay.

Nest Maintenance:

To assess if there was a division of labor in nest maintenance, we introduced a nest contaminant (a small rock) into the terra cotta pot serving as the focal pair's nest, and measured behaviors aimed at removing the contaminant from the nest (strikes, bites, and latency).

We did not find a significant difference between males and females in any of the recorded behaviors in nest maintenance (All P >0.05). Males and females produced remarkably similar counts of behaviors, while latency differed slightly more by sex, though not significantly.

We did however find a significant effect of egg condition on a several recorded behaviors. This included the sum of behaviors, (No transformation, $F_{2,128} = 16.245$, $P<0.001$, $\eta^2_p = 0.20$; Fig. 4 C), strikes (No transformation, $F_{2,128} = 16.165$, $P<0.001$, $\eta^2_p = 0.20$), bites (No transformation, $F_{2,128} = 5.806$, $P = 0.004$, $\eta^2_p = 0.08$), and latency (No transformation, $F_{2,128,}$ $P<0.001$, $\eta^2_p = 0.17$; Fig. 4 D). As in the small intruder aggression assay, pairs were significantly more aggressive toward the contaminant with eggs in the nest (own or surrogate) as opposed to without. Pairs responded to the contaminant 63% faster with eggs in the nest compared to without, and produced nearly double the number of aggressive behaviors including a 2-fold increase in the number of strikes and a 1.7-fold increase in bites (see Supplementary Excel File 3 for pairwise comparisons, 16 for ANOVA tables, and 17 for means and standard errors).

There was also a significant interaction between egg condition and sex for latency (No transformation, $F_{2,128} = 7.461$, $P<0.001$, $\eta^2_p = 0.10$; see Supplementary Excel File 3 for pairwise comparisons). Interestingly, males showed a significantly higher latency to address the contaminant in the no eggs condition than in the own or surrogate conditions, while females showed no such difference. This may be caused by the fact that males are less likely to enter the nest when there are no eggs to be cared for leading to lower latency, while females, who are not the primary caregivers, do not differ across egg conditions.

Once again, we found a significant effect of round on bites (No transformation, $F_{2,128} = 4.592$, $P = 0.012$, $\eta^2_p = 0.07$; Fig. 4 E) and latency (No transformation, $F_{2,128} = 3.984$, $P = 0.021$, $\eta^2_p = 0.06$; Fig. 4 F). As rounds progressed, pairs got more aggressive including a 1.8-fold increase in bites, and a 50% decrease in latency between round 1 and round 3 (see Supplementary Excel File 3 for pairwise comparisons, 16 for ANOVA tables, and 17 for means and standard errors).

We also found a significant interaction between sex and round on the sum of behaviors (No transformation, $F_{2,128} = 3.290$, $P = 0.040$, $\eta^2_p = 0.05$), strikes (No transformation, $F_{2,128,}$ $P = 0.008$, $\eta^2_p = 0.07$), and latency (No transformation, $F_{2,128} = 4.355$, $P = 0.015$, $\eta^2_p = 0.06$; see Supplementary Excel File 3 for pairwise comparisons). Females tended to get more aggressive as rounds progressed, while males largely did not.

We found that all of the recorded behaviors were significantly repeatable (see table 8 for ICC and P-values).

Modeled Behavior	Model ICC	P-Value of Random Effect
Sum of Behaviors	0.575	**<0.001**
Latency	0.266	**<0.001**
Strikes	0.489	**<0.001**
Bites	0.450	**<0.001**

Table 8. Model ICC and P-value of the random effect for all modeled behaviors in the nest maintenance assay.

Principal Component Analysis (PCA)

In addition to analyzing each behavior separately to determine effects of sex, round and egg condition, we also sought to evaluate how a composite of all of the recorded assays might be a useful metric for differentiating the sexes. Thus, we performed a PCA analysis on the mean of the sum of behaviors from each assay collapsed across all rounds and egg conditions within individuals. We found that the first and second PCs explained 65.7% of the variation. A plot of PC2 on PC1 shows the individuals largely separate based on a parental-aggression axis (Fig. 5) which effectively separates the sexes, except for a few individuals at the margins. These include 1 male (T18M in Fig. 5 A) which displays similar levels of aggression and parental care as females, and 2 females that do more parenting and display less aggression than usual (A24F and B22F in Fig. 5A). PC1 from this composite PCA analysis significantly differentiated the sexes ($t(16) = 3.512$, $P = 0.003$), whereas PC2 did not ($P>0.05$; see Supplementary Excel File 18 for statistical analyses). Additionally, we found that PC2 captured the variation in aggression assays. Conspecific-oriented aggression assays loaded negatively, while non-conspecific aggression assays loaded positively (with the exception of immediate reaction to a threat), suggesting conspecific aggression and non-conspecific aggression constitute two distinct types of aggression.

In addition, we performed multiple separate PCA analyses for all of the recorded behaviors within each assay (supplementary Figs. 1-3). We then calculated the Euclidean distance between the mean male and mean female PC1 and PC2 coordinates. Based on this metric, the assays that best differentiated the sexes were parental care (2.767), female-oriented aggression (2.645), and large intruder aggression (2.297) (table 9). PC1 extracted from the analyses of measures of parental care, large intruder aggression, female-oriented aggression, and immediate reaction to a

threat significantly differentiated males and females, but not from the other assays, while PC2 extracted from male-oriented aggression significantly differentiated the sexes (see Supplementary Excel File 18 for statistical analyses). When analyzing each assay separately, a few consistent patterns emerge. As in our statistical analysis, in parental care, males are characterized by high levels of parental behaviors and time spent in the nest (supplementary Fig. 1). In non-conspecific aggression assays (large intruder, small intruder, immediate reaction, nest maintenance), males differentiate from females based on high latency and low direct aggression (bites, strikes, chases/charges) (supplementary Figs. 1-3). Similarly, in conspecific-oriented aggression assays, males differentiate from females based on direct versus indirect aggression, with males characterized by high indirect aggression, and females characterized by high direct aggression, while latency does not differentiate the sexes (supplementary Fig. 2).

Assay	Distance
Parental Care	2.767
Small Intruder Aggression	1.165
Large Intruder Aggression	2.297
Male-Oriented Aggression	1.565
Female-Oriented Aggression	2.645
Immediate Reaction to a Threat	2.147
Nest Maintenance	0.577

Table 9 Distances. Parental care, female-oriented aggression, and large-intruder aggression are the best at separating males and females.

Pair Dynamics

To determine whether the male-female pairs coordinated their behaviors toward the introduced stimuli, we estimated Pearson's correlations separately for each assay using the sum of behaviors collapsed across round and egg conditions. The only correlation that was significant was female-oriented aggression where levels of aggression in the male and female of the pair were positively correlated ($r(7)= 0.720$, $P = 0.029$) (supplementary Fig. 4).

Behavioral Syndromes

To assess the presence of behavioral syndromes, we fit Pearson's correlations in a pairwise manner between all 7 behavioral assays. For this analysis, we used the mean of sum of behaviors collapsed across round and egg condition to represent individual values for each assay. This resulted in a total of 21 comparisons. Because we observed several significant effects of sex, we assessed behavioral syndromes for males and females separately.

Males

Of the 21 correlations calculated for males, 5 produced a significant outcome. Large intruder aggression was significantly positively correlated with small intruder aggression ($r(7) = 0.710$, $P=0.032$; Fig. 6A). Both female-oriented aggression and male-oriented aggression were significantly positively correlated with immediate reaction to a threat ($r(7) = 0.897$, $P=0.001$;Fig. 6B; $r(7)=0.831$, $P=0.006$; Fig. 6C, respectively). Female-oriented aggression was also positively correlated with male-oriented aggression ($r(7)=0.819$, $P=0.007$; Fig. 6D), and negatively correlated with parental care ($r(7)=-0.688$, $P=0.041$; Fig. 6E).

Females

Surprisingly, of the 21 correlations we calculated for females, only 1 was significant. Small intruder aggression was significantly correlated with nest maintenance ($r(7)=0.685$, $P=0.042$) (Fig. 6F). This is perhaps unsurprising due to the high visual similarity between a hermit crab in the small intruder aggression assay and a rock in the nest maintenance assay, though it is unclear why this syndrome is detectable in females but not males.

Taken together, these data show that males display several behavioral syndromes (male-oriented aggression positively correlated with immediate reaction to a threat, male and female-oriented aggression were positively correlated, and female-oriented aggression was negatively correlated with parental care). In contrast, females only showed one correlation that is arguably marginally a syndrome because of the similarity in the measurements between small intruder aggression and nest maintenance.

Discussion

Here we significantly broadened the range of quantifiable behaviors in *A. ocellaris*, enabling a more comprehensive examination of their unique characteristics in a laboratory setting. To this end, we established significant individual differences, effects of sex, effects of the environment (e.g., whether or not eggs are present), and effects of repeated testing (e.g., round) on multiple measures of territory defense, aggression, and parental care. All assays contained measures that were significantly repeatable across contexts and rounds. The average ICC of our significantly repeatable behaviors was 0.37, consistent with average estimates of repeatability of behavior (Bell et al., 2009). Interestingly, in male and female-oriented aggression, latency was not significantly repeatable. This lack of repeatability could be due to a significant effect of the behavior of the stimulus animal, or spontaneity in the decisions to engage. The female-male aggression ratio was also not significantly repeatable which is likely due to its compound nature combining two partially repeatable behaviors: male and female-oriented aggression.

We found that the best assays for detecting sex differences were parental care, large intruder aggression and female-oriented aggression. These assays detected large, and significant sex effects across environmental conditions and rounds. However, we found that none of these, whether considered separately or combined into a linear combination could perfectly distinguish the sexes. This was due to strong and repeatable individual differences in behavior with some females behaving similarly to males (e.g., B22F in Fig.5A and supplementary Figs. 1C, 2A, C, and 3A), and some males behaving similarly to females (e.g., T18M in Fig. 5A and supplementary Figs. 1A, C).

While prominent sex differences were detected for most of the assays, small intruder aggression, nest maintenance were exceptions. One explanation is that in small intruder aggression and nest maintenance, the crab and rock were placed in or around the terracotta pot which serves as the nest, drawing more attention from the male, equaling the effort from the female. Thus, while these behaviors may be useful for establishing individual differences, they are poor sex discriminators. Consistent with previous reports, we also did not find significant sex differences in male-oriented aggression (Parker et al., 2022).

In addition to uncovering large sex differences for independent, repeatable behaviors, we also observed strong evidence for sex specific behavioral syndromes in *A. ocellaris*. In males, we observed positive correlations between several aggression measures and boldness, and a negative correlation between aggression and parental care. However, in females, only one correlation was observed for aggression measures in nest maintenance and small intruder aggression. Most of the sex differences we observed including the syndromes are likely caused at some level by the organizational and activational effects of the sex steroid hormones 11-ketotestosterone (11-KT) and estradiol (E2), which have been shown to have broad effects on multiple aspects of physiology and behavior (Gegenhuber et al., 2022; Godwin, 2010; Stennette & Godwin, 2024). Additionally, it may be the case that females are more variable within sex than males, resulting in our statistical analyses not being able to detect behavioral syndromes in females as well as males given the small sample sizes.

In anemonefish, male-female pairs work together as a reproductive unit (Laudet & Ravasi, 2022), yet our study shows that an individual's behavior was largely independent of the behavior of its partner, with the exception of female-oriented aggression. This is likely because of the strong division of labor, where individuals perform their duties without much help or compensation from the other (R. DeAngelis et al., 2017). The correlation between aggression levels displayed by males and females in the pair toward the stimulus female could reflect the size differential between males and females. A male is probably unwilling to be aggressive towards a much larger female unless its female partner is also engaging with her for fear of retaliation.

Interestingly, in female-oriented aggression, the sexes did not significantly differ in the sum of aggressive behaviors, but did differ in their behavioral composition, with males preferring indirect aggression (displays) while females preferred direct aggression (bites) (Figure 3 C, D). Thus, future studies that use this assay to evaluate sex differences should evaluate displays and bites separately. The different ways in which males and females direct aggression towards a female intruder is likely due to the significant size difference between males and females and their unique natural history. Males may be less willing to directly confront a female because of possible injury, whereas a female will fight to the death to maintain / usurp the α position in the hierarchy as searching for a new anemone means almost certain death (Da Silva & Nedosyko, 2016; Iwata & Manbo, 2013).

One of the goals of the study was to expand the behavioral assays available for measuring aggression and territorial defense levels, particularly inter-specific aggression, with the hope it might better differentiate the sexes. Here, we show for the first time significant and repeatable sex differences in the large intruder assay, where a single damselfish is introduced into the tank and aggressive behaviors are recorded for 10 minutes. This is slightly different from a previous study where we used 3 damselfish (R. DeAngelis et al., 2020). When 3 damselfish are used, there is

no significant sex difference (R. DeAngelis et al., 2020), but when only one is used, a significant sex difference emerges, with females displaying 3.5-fold more aggressive acts than males. The difference is likely because one fish can be targeted whereas 3 fish overwhelm the pair in the confines of the aquarium.

In all of our assays except parental care, we found at least one behavior that significantly changed as rounds progressed. This was almost always an increase in aggressive behavior or a decrease in latency which indicates an increase in boldness. This increase in aggression was likely caused by acclimation to the stimulus animal increasing the fish's confidence that aggression does not pose a risk. We likely did not find any effect of round in parental care because parental care is something fish do repeatedly in our lab, while exposure to the stimuli (e.g., damselfish, rock, crab, novel anemonefish) is a relatively rare experience for our fish. In male and female-oriented aggression, the increase of aggression as round progressed may also be caused by winner effects as, in most cases, the focal pair would likely perceive they 'won' the encounter (Dijkstra et al., 2012; Dugatkin, 1997; Hsu & Wolf, 1999).

We also found in several assays that the fish's behavior depended on whether there were eggs in the nest or not. In small intruder aggression and nest maintenance assays, pairs were more aggressive when there were eggs in the nest but did not distinguish whether their eggs were their own or surrogate, consistent with previous studies (Phillips et al., 2020). However, in in male and female-oriented aggression, pairs were significantly less aggressive in the own condition relative to the none or surrogate conditions. The lower levels of aggression when caring for their own eggs relative to no eggs could reflect a trade-off between aggression and parental care (R. DeAngelis et al., 2020). However, the difference between own and surrogate is unexpected, and the explanation is unclear. This difference is not likely to be due to hormonal differences in the two conditions since in the surrogate condition, the pairs own eggs were replaced with surrogate eggs of a similar age only after they had spawned, ensuring that pairs were at the same point in the spawning cycle. The small effect size between aggression in the two conditions show that if pairs can distinguish their own eggs from surrogate eggs, their behaviors toward those eggs are exceedingly similar.

One potential use of the behavioral assays described herein that produce significant sex differences in behavior is the study of protandrous sex change in anemonefish. Over the course of sex change, fish destined to become females maintain a male behavioral phenotype until late in sex change, when they adopt a female behavioral phenotype (Parker et al., 2022). Thus, behavioral assays are useful for studying sex changing fish by quantifying the 'behavioral sex' of the fish during its progression through sex change. We suggest that future studies studying sex change use both the parental care assay, an assay that quantifies 'male-like' behaviors, with the large intruder aggression assay, an assay that quantifies 'female-like' behavior, territorial aggression, in order to capture the breadth of sex differences along the parental care-aggression axis (Fig. 5A). We suggest the large intruder aggression assay over the female-oriented aggression assay and immediate reaction to a threat assay due to its large effect size (females produced 3.5 times the number of aggressive behaviors of males) and low overlap between males and females (Fig. 2B) relative to the other assays mentioned. All the behavioral assays described herein, whether they showed sex differences or not showed repeatable individual differences, effects of round or egg condition and thus could be useful for studying neurological mechanisms of individual differences in these behaviors (R. DeAngelis et al., 2017, 2020).

In conclusion, we measured the behavior of male and female *A. ocellaris* pairs in 6 novel behavioral assays and 1 established parental care assay. All behaviors were measured across 3 contexts that varied depending on whether no eggs, own or surrogate eggs were present. In addition, all behaviors were measured 3 separate times during separate spawning cycles to evaluate repeatability. 34 out of 38 behaviors showed significant repeatability. The best assays for differentiating the sexes were the parental care assay, large-intruder aggression assay, and female-oriented aggression assay. Additionally, we found consistent differences in territorial aggression depending on egg condition and level of repetition, where pairs tended to be more aggressive without eggs, and tended to increase aggression with increased exposure to the stimulus. Further, we found that the behaviors of males and females within a pair were largely uncorrelated, except for female-oriented aggression. Interestingly, males displayed several behavioral syndromes, but females only displayed one. This study significantly broadens the number of laboratory behavioral assays available for studying female behavioral dominance and territoriality, protandrous behavioral sex change, behavioral plasticity and the neuroendocrine basis of parental care and aggression in the iconic anemonefish, *A. ocellaris*.

Author Contributions

Gabriel Graham: Conceptualization, Methodology, Formal analysis, Data curation, Writing - Original draft, Writing – Review and editing, Visualization. **Isabella Wilton:** Writing - Original draft, Data curation. **Emily**

Panczyk: Data curation. **Justin Rhodes:** Conceptualization, Methodology, Formal analysis, Writing - Original draft, Writing – Review and editing, Visualization, Supervision, Project administration, Funding acquisition.

Data Availability

Data are available as supplementary material.

Declaration of Competing Interest

No competing interests

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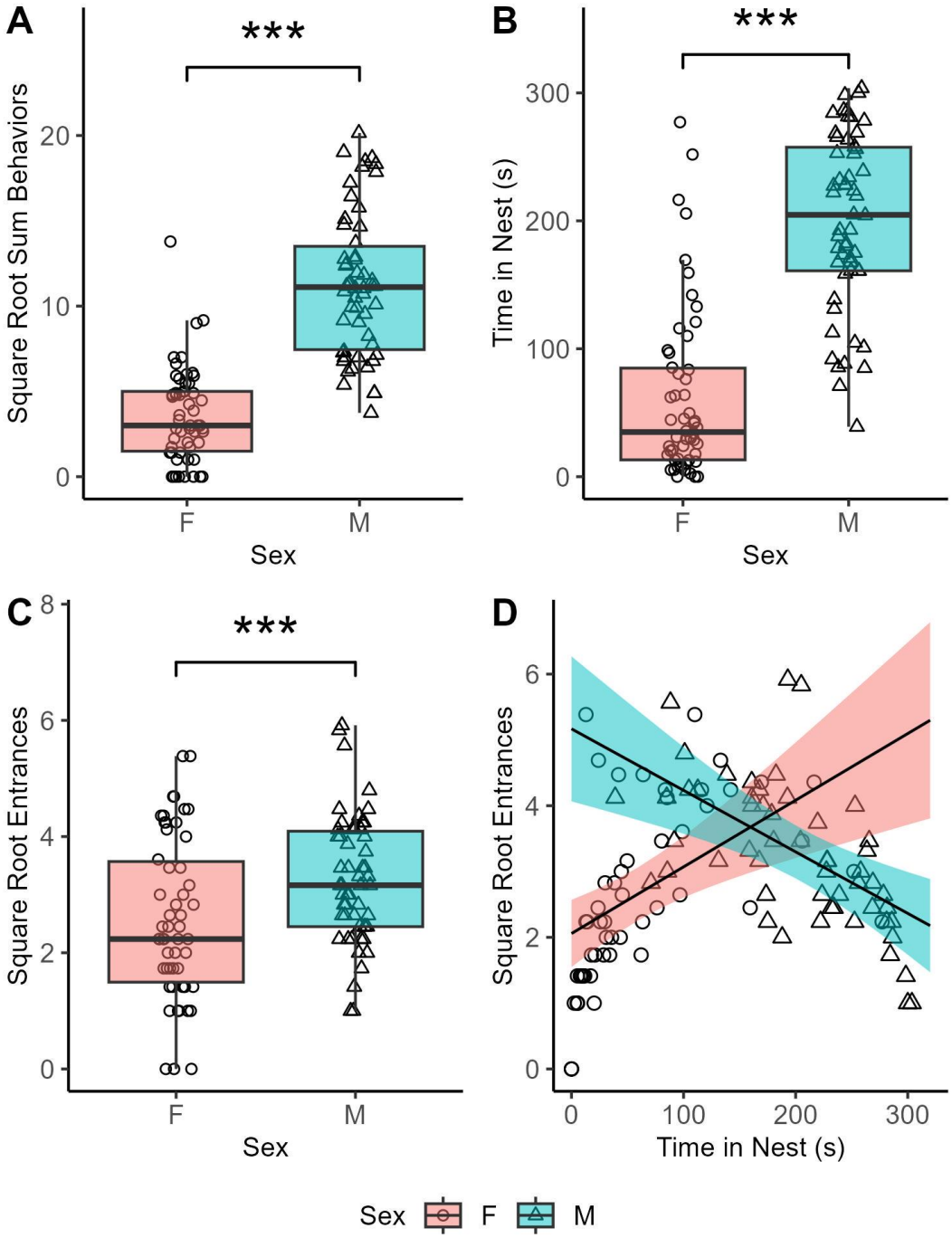

Figure 1. Males are the primary caregivers of the eggs. All datapoints are shown including repeated measures across the 2 egg conditions and 3 rounds A) The square root of the sum of parental behaviors by sex. B) The amount of time spent in the nest by sex. C) The square root of the number of nest entrances by sex. D) Square root entrances plotted against time spent in nest, split by sex. Males show a negative correlation whereas females show a positive correlation. In A-C, boxplots display the inter quartile range (IQR), the horizontal line represents the median, and whiskers extend to 1.5 times the IQR. In D, the line represents the linear regression estimate and ribbon represents +/- 95% confidence interval (CI) "***" Indicates $P<0.001$, from the mixed model with individual entered as a random effect (see methods).

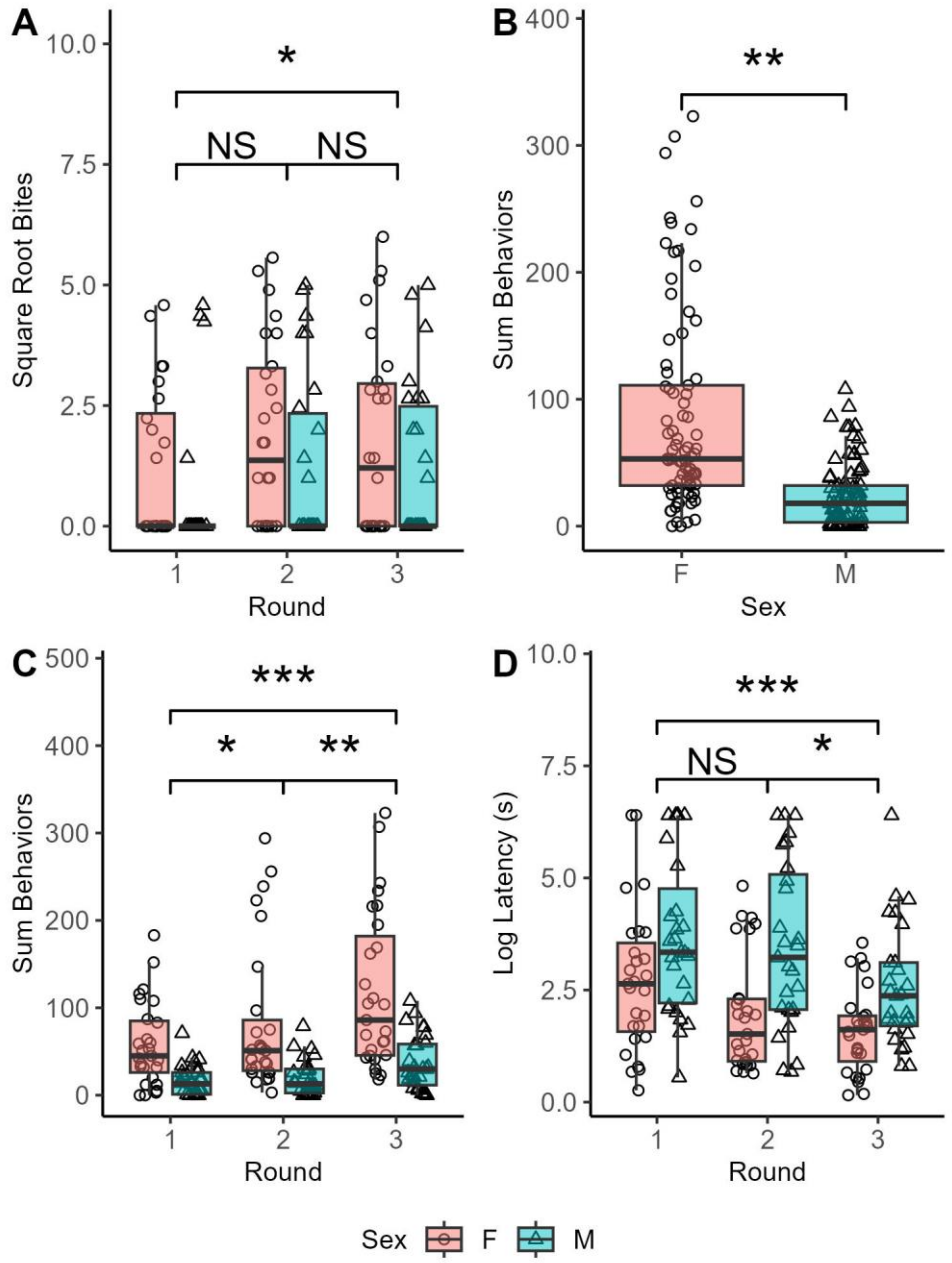

Figure 2. Females are more aggressive to a large intruder than males, and aggression to a large or small intruder increases as rounds progress. All datapoints are shown including repeated measures. A) Square root of bites in small intruder aggression, shown by round and sex. Pairs got more aggressive as rounds progressed. B) Sum of aggressive behaviors in large intruder aggression shown by sex. Females were significantly more aggressive than males. C) Sum of aggressive behaviors in large intruder aggression by round and sex. Pairs got more aggressive as rounds progressed, especially females. D) Log of latency to address the stimulus animal in large intruder aggression by round and sex. Pairs addressed the intruder more quickly as rounds progressed. Boxplots display the IQR, the horizontal line represents the median, and whiskers extend to 1.5 times the IQR. "***" Indicates $P<0.001$, "**" indicates $P<0.01$, and "*" indicates $P<0.05$, "NS" indicates no significant difference.

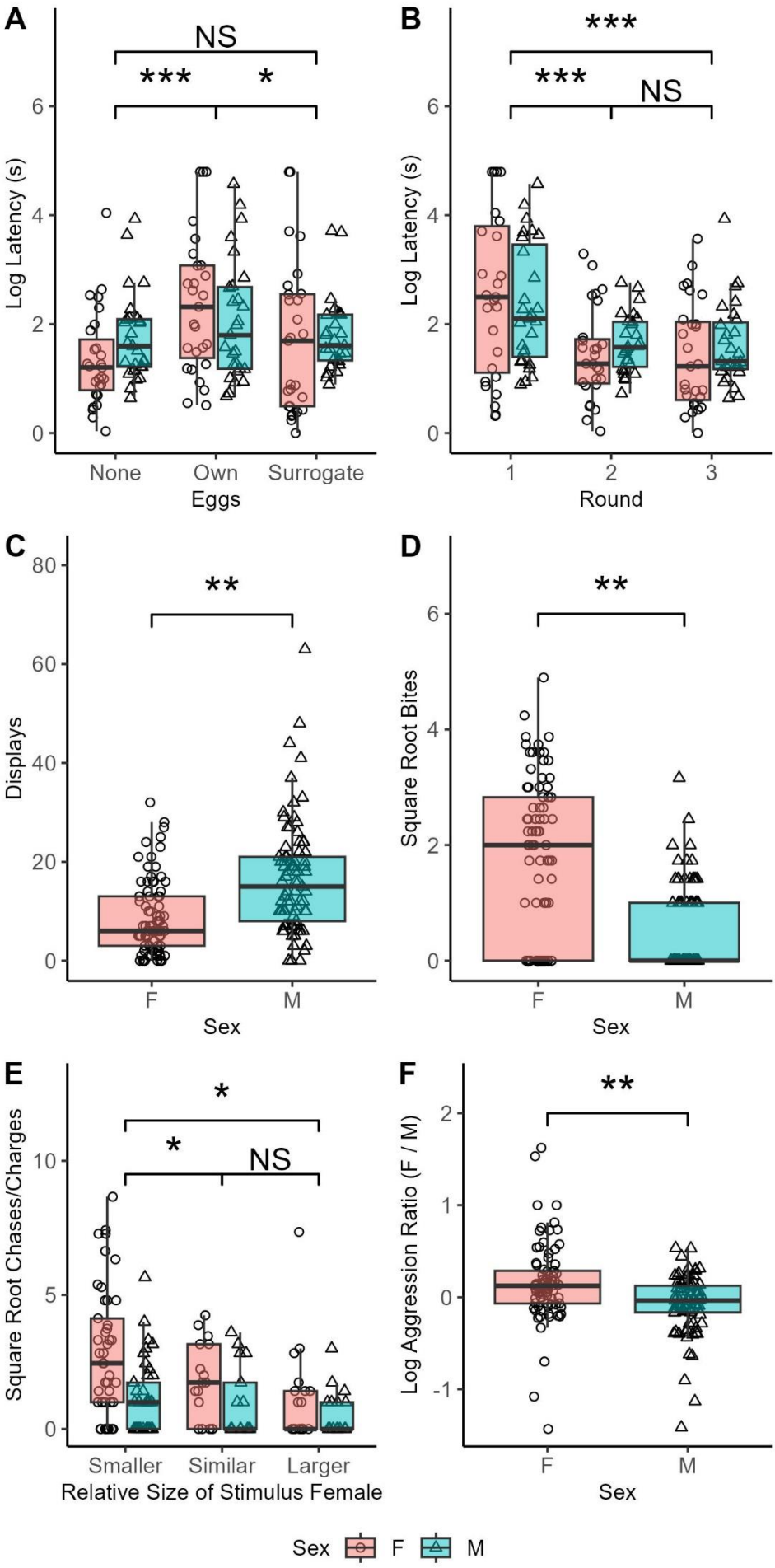
A
NS
***
*
Log Latency (s)
None
Own
Surrogate
Eggs
B
***
***
NS
Log Latency (s)
Round
C
**
Displays
Sex
D
**
Square Root Bites
Sex
E
*
*
NS
Square Root Chases/Charges
Smaller
Similar
Larger
Relative Size of Stimulus Female
F
**
Log Aggression Ratio (F / M)
Sex
Sex
F
M

Figure 3. Sex differences in female-oriented aggression are apparent when displays and bites are separated, but no sex differences in male-oriented aggression are detected. All datapoints are shown including repeated measures. A) Log latency to address the stimulus male in male-oriented aggression shown by egg condition and sex. Pairs were significantly faster to respond to the intruder male when there were no eggs in the nest as compared to when they had their own or surrogate eggs in the nest. B) Log latency to address the stimulus male in male-oriented aggression assay shown by round and sex. Pairs responded to the stimulus male significantly faster as rounds progressed. C) Total number of displays toward the stimulus female in female-oriented aggression shown by sex. Males display at females more than females. D) Square root of the total number of bites toward the stimulus female in female-oriented aggression shown by sex. Females bite females more than males. E) Square root of chases/charges by size of the stimulus female relative to the focal female and sex in female-oriented aggression. Pairs were more aggressive toward similarly sized or larger stimulus females compared to smaller stimulus females, but do not discriminate between similar and larger stimulus females. F) Log female-oriented aggression /male-oriented aggression ratio shown by sex. The ratio is higher in females than males. Boxplots display the IQR, the horizontal line represents the median, and whiskers extend to 1.5 times the IQR. "***" Indicates $P<0.001$, "**" indicates $P<0.01$, and "*" indicates $P<0.05$, "NS" indicates no significant difference.

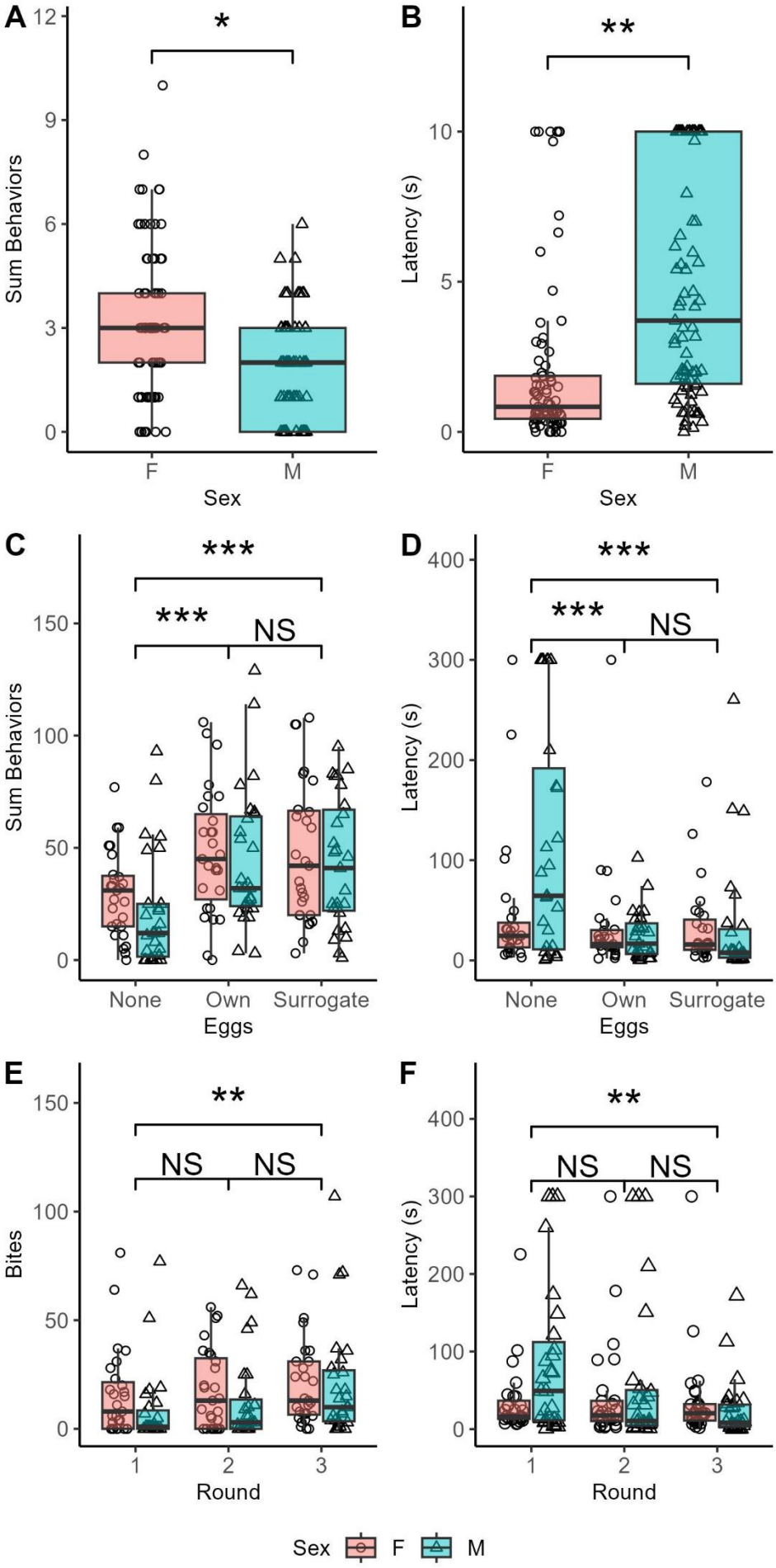
A
B
C
D
E
F
Sum Behaviors
Latency (s)
Bites
Sex
F
M
None
Own Eggs
Surrogate
Round
1
2
3
*
**
***
NS
Sex F M

Figure 4. Females are bolder than males but share equally in nest maintenance. All datapoints are shown including repeated measures. A) Sum of aggressive behaviors in immediate reaction to a threat shown by sex. Females more aggressive in a short timeframe than males. B) Latency in immediate reaction to a threat by sex. Females responded to the threat more rapidly than males. C) Sum of aggressive behaviors in nest maintenance shown by egg condition and sex. Pairs were more aggressive toward the contaminant when they had surrogate or own eggs in the nest as compared to no eggs. D) Latency to address the contaminant in nest maintenance by egg condition and sex. Pairs took longer to address the contaminant when no eggs were present, especially males. E) Total number of bites in nest maintenance shown by round and sex. Bites increased as rounds progressed. F) Latency in nest maintenance by round and sex. Latency decreased as rounds progressed, especially for males. Boxplots display the IQR, the horizontal line represents the median, and whiskers extend to 1.5 times the IQR. "***" Indicates $P<0.001$, "**" indicates $P<0.01$, and "*" indicates $P<0.05$, "NS" indicates no significant difference.

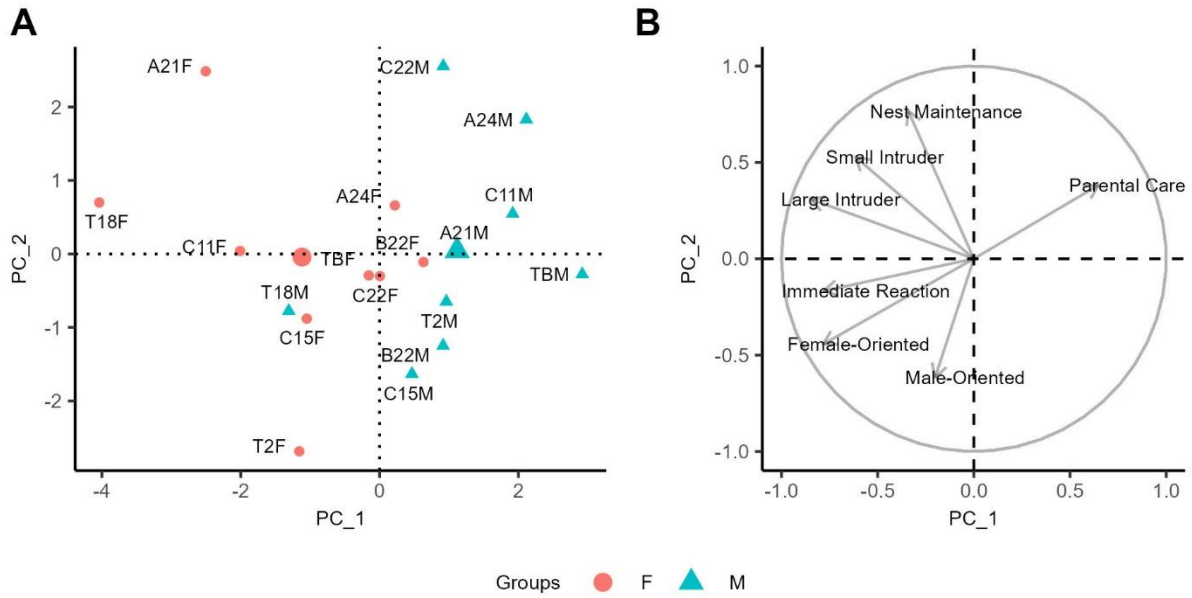

Figure 5. Males and females separate largely along a parental care - aggression axis. A) A PCA was performed on the sum of behaviors (collapsed across rounds and egg conditions) for each of the 7 behavioral assays. PC1 is plotted against PC2 showing males and females separately. In addition, the global female and male averages are shown as large red circle and large blue triangle respectively. B) Loadings of the corresponding assays are shown on PC1 and PC2. Males and females largely split along a parental care - aggression axis, where parental care loads to quadrant 1 while the various aggression assays load to quadrants 2 and 3.

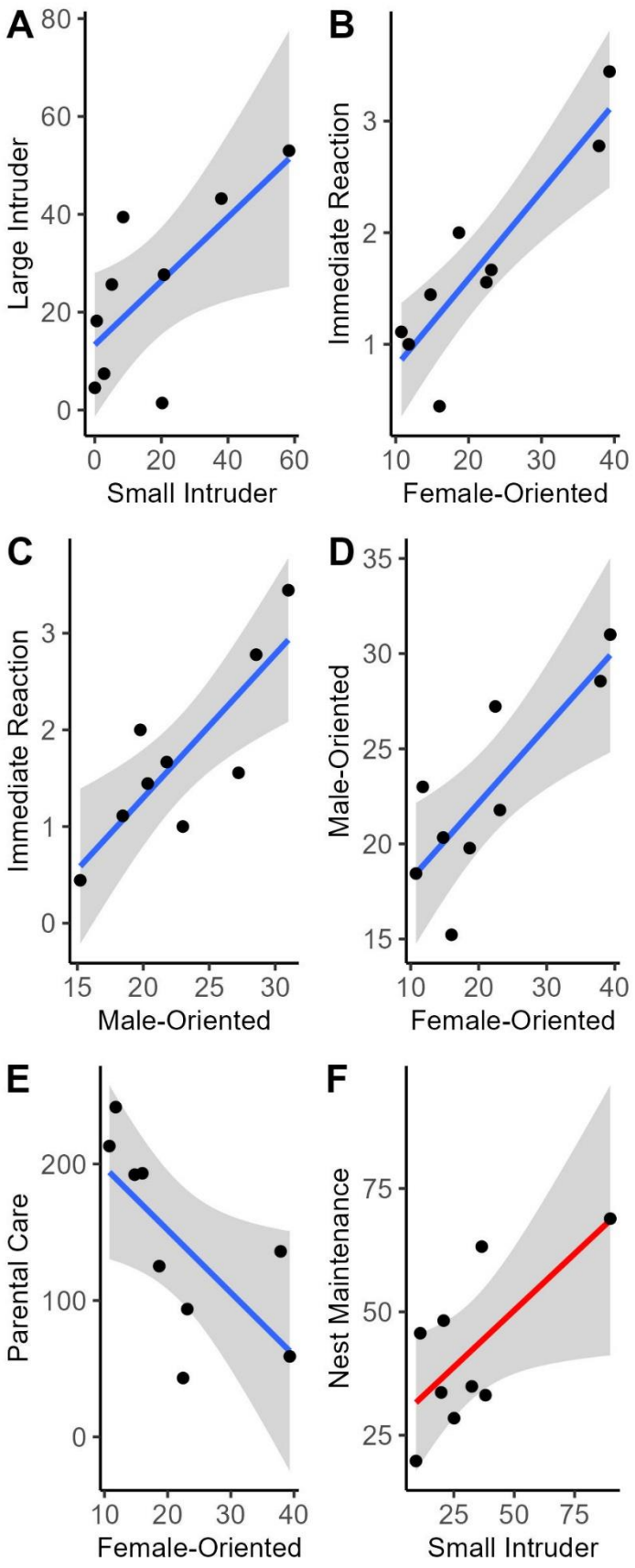
A
Large Intruder
Small Intruder
B
Immediate Reaction
Female-Oriented
C
Immediate Reaction
Male-Oriented
D
Male-Oriented
Female-Oriented
E
Parental Care
Female-Oriented
F
Nest Maintenance
Small Intruder

Figure 6. Males display several behavioral syndromes, while females display only one. All data points represent the sum of behaviors collapsed across round and egg condition within each individual A) Sum of aggressive behaviors in large intruder aggression plotted against sum of aggressive behaviors in small intruder aggression, males only. B) Sum of aggressive behaviors in immediate reaction to a threat plotted against the sum of aggressive behaviors in female-oriented aggression assay, males only. C) Sum of aggressive behaviors in immediate reaction to a threat plotted against sum of aggressive behaviors in male-oriented aggression, males only. D) Sum of aggressive behaviors in male-oriented aggression plotted against sum of aggressive behaviors in female-oriented aggression, males only. E) Sum of parental care behaviors) plotted against sum of aggressive behaviors in female-oriented aggression, males only. F) Sum of behaviors in nest maintenance assay plotted against sum of aggressive behaviors in small intruder aggression, females only. Blue and red lines represent simple linear regression lines for males and females, respectively.