Nonapeptides mediate trade-offs in parental care strategy

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\textbf{ABSTRACT}

Parental care represents a suite of distinct behaviors performed by parents to maximize fitness. Dynamic shifts in parental care behaviors, such as between nest defense and direct provisioning of the offspring, are required in response to environmental variation. However, the neural mechanisms which mediate such behavioral shifts remain a mystery. The anemonefish, \textit{Amphiprion ocellaris}, represents an experimentally valuable model in social neuroscience which is conducive to manipulating the environment while simultaneously measuring parental care. The goal of this study was to determine the extent to which arginine vasotocin (AVT) and isotocin (IT) signaling are necessary for males to shift between direct egg care and aggressive nest defense in the presence of intruders, Domino damselfish (\textit{Dascyllus trimaculatus}). The IT receptor antagonist \textit{desGly-NH\textsubscript{2}}-d(CH\textsubscript{2})\textsubscript{5}[D-Tyr\textsuperscript{2},Thr\textsuperscript{4}]AVT, significantly reduced direct egg care, while at the same time increased levels of aggressive nest defense relative to vehicle. Conversely, blockade of AVT using the antagonist \textit{d(CH\textsubscript{2})\textsubscript{5}[Tyr(Me)\textsuperscript{2}]AVP}, reduced aggression and tended to increase egg care. Results demonstrate that male anemonefish alter their parental strategy in response to allospecific intruders, and that IT and AVT signaling oppositely regulate parental care displays of aggression versus egg care.

1. Introduction

Parental care encompasses a suite of behaviors performed by parents to promote the survival of their offspring, often at a cost to both their own survival and future reproductive opportunities (Badyaev and Ghalambor, 2001; Clutton-Brock, 1991; Gross, 2005; Robert, 1972; Royle et al., 2012). Examples of parental care include directly providing resources for offspring, (e.g. providing food, grooming, and egg care), as well as indirect parental care (e.g. burrowing, nest building and the aggressive defense of offspring from predators). Hence, parental care is inherently complex, as parents must deal with multiple simultaneously competing demands (Royle et al., 2014). Parental behaviors are therefore inherently plastic, and the behaviors individuals display are context and situational dependent (O’Rourke and Renn, 2015; Winkler, 1987). Variability in brood size (Rauter and Moore, 2004; Wendeln and Becker, 1999), parental condition (Velando and Alonso-Alvarez, 2003), partner investment (Wright and Cuthill, 1989), individual experience (Nunes et al., 2001), and predation risk (Fontaine and Martin, 2006), can all influence variability in the allocation of parental investment in offspring. For example, a sand goby (\textit{Pomatoschistus minutus}) father robustly cares for his brood to promote offspring development, yet when predators arrive he must vigorously guard the nest (Lissåker and Kvarnemo, 2006). Furthermore, in a study including 5 different species of biparental birds, males frequently visit nests to provide food to incubating females. However, these visits can be risky as they cue predators to the location of the nest. When perceived predation risk increases, males visit the nest less frequently, weighing the costs of reduced offspring nourishment against the benefit of reduced predation (Ghalambor and Martin, 2002). Taken together, these studies highlight the dynamic nature of parental care, where it is critical for individuals to display the appropriate behavior to maximize fitness. The dynamic behavioral changes parents exhibit during offspring care must be mediated by plastic mechanisms within the brain. In order to identify these mechanisms, studies exploring concurrent behaviors with varying ecological demands that necessitate the adaptive behavioral choices are needed.

While the theoretical application and evolutionary consequences of parental care trade-offs have been extensively discussed (Baylis, 1981; Clutton-Brock, 1991; Gross, 2005; Gross and Sargent, 1985; Royle et al., 2012), much remains to be learned about the neural mechanisms necessary for switching between parental displays. The identification of nonapeptides and their broad role in social behaviors presents an ideal starting point in understanding the mediation of discrete behavioral displays. Nonapeptides represent a group of ancient evolutionarily
conserved neurohormones synthesized primarily within the preoptic area (POA) of the hypothalamus as vertebrate lineages (Goodson, 2008; O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012). This family of peptides was generated from a gene duplication and includes the non-mammalian vasopressin homolog vasotocin (AVT), and the OT homologs: mesotocin (MT-birds and reptiles) and isotocin (IT-fish) (Goodson and Bass, 2001; Goodson and Thompson, 2010; Keverne and Curley, 2004; O'Connell and Hofmann, 2011). Not only are the peptides conserved, but peptide receptors for both AVP and OT like homologs are pervasive across regions of the social decision-making network (SDMN), a highly conserved neural network of brain regions which function in the regulation of social behavior (O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012). Moreover, patterns of neural projections throughout the brain are also remarkably similar (Donaldson and Young, 2008; Goodson, 2005; Goodson and Bass, 2000; O'Connell and Hofmann, 2011; Thompson and Walton, 2009), suggesting that these nonapeptide signaling pathways have remained relatively unchanged over 450 million years of vertebrate evolution. Hence, exploration of the neural regulation of parental behavior outside of non-model systems may provide valuable insight into the evolutionarily conserved function of this ancient nonapeptide family.

Research across a wide range of taxa has repeatedly implicated nonapeptides as key components in the regulation of social behavior (Albers, 2015; Goodson and Bass, 2000, 2001; Santangelo and Bass, 2006). While sex and species-specific differences are present, some common threads have emerged. OT/IT has been widely implicated as important for social salience and parental care (Bartz et al., 2010; Donaldson and Young, 2008; Gubernick et al., 1995; Olazabal and Young, 2006; Oldfield and Hofmann, 2011; Saito and Nakamura, 2011). In mammals, the release of OT at parturition is critical for a variety of physiological processes as well as influencing behavioral changes important for mother-infant bonding (Bartz et al., 2010). Furthermore, the role of OT/IT is not limited to mammals nor specific to female parental care. Studies in fish reveal IT signaling is conserved in the promotion of parental care in both sexes; blockade of IT during paternal care reduces paternal effort in multiple species (DeAngelis et al., 2017b; O'Connell et al., 2012). Studies which have examined the role of AVP/AVT suggest its function in the brain depends on the specific social and ecological pressures within each species, but broadly appears to promote behaviors which increase reproductive opportunities (Ramshad et al., 1994; Fehm-Wolfsdorf et al., 1988; Ferris, 1992; Sensar et al., 2001). AVP has been extensively studied in pair bonding roles where the distribution of AVP receptors has a strong relationship with social mating systems (Donaldson and Young, 2008). AVP/AVT has repeatedly been implicated as important for aggression, nest defense, and intraspecific aggressive interactions in dominance hierarchies (Greenwood et al., 2008; Kleszczyńska et al., 2012; Yaeger et al., 2014). While the role of AVP/AVT in parental care is less clear, several studies have implicated AVP/AVT as important for parental aggression, including displays of vigilance and nest defense (Bosch and Neumann, 2010; DeAngelis et al., 2017b; Marler et al., 1995; Schulte and Summers, 2017; Ten Eyck and ul Haq, 2012).

In order to understand how nonapeptide systems influence trade-offs in parental behaviors, specifically switching between direct provisioning of offspring and aggressive offspring defense, study systems are needed in which the environments can be easily manipulated, and parents forced to adjust parental displays in response to dynamic environments. The anemonefish A. ocellaris is an emerging model system within social neuroscience that provides an opportunity to manipulate the risk of nest predation in a semi-naturalistic way. Moreover, several life history characteristics make this a felicitous laboratory study system for identifying neural mechanisms of parental care trade-offs. Due to their obligate symbiosis with protective host sea anemones they live in relatively small, simple and isolated social groups and have unusually small home ranges (restricted with within a meter of the host anemones). As a consequence, they are extremely aggressive in the defense of their territory and nest. Furthermore, males of this species display an extraordinary amount of paternal effort; paternal behavior is consistently variable between individuals across spawning periods and easily quantified from video recordings (DeAngelis et al., 2017b).

In a previous report, we demonstrated that blockade of IT signaling (using a specific IT receptor antagonist) during high levels of paternal care in A. ocellaris dramatically reduced the amount of egg care (nipping and fanning of the eggs) (DeAngelis et al., 2017b). Conversely, when males were administered an AVT-receptor antagonist, the amount of egg care increased. Previous reports have highlighted the important role of AVT signaling during aggressive interactions (Hattori and Wilczynski, 2009; Huffman et al., 2015; Ramallo et al., 2012; Yaeger et al., 2014). Hence, we hypothesized that the increase in paternal effort in response to the AVT-receptor antagonist is a consequence of a reduction in vigilance and nest defense, thereby allowing the animal to allot more effort towards direct measures of parental care (nipping and fanning of the eggs). However, there were no nest predators in that study, and thus it was not possible to conclude that the increase in egg care from the AVT receptor antagonist was associated with reduced vigilance.

The goal of this study was to determine whether AVT and IT signaling are necessary for males to modify their levels of direct egg care versus egg defense in the presence of allospecific nest intruders. To accomplish this, we modified the experimental paradigm by introducing Domino damselfish (Dascyllus trimaculatus) into the territory of an anemonefish pair at a time when the male is displaying peak levels of parental care towards their batch of eggs. We hypothesized that introducing the damselfish would reduce the amount of effort directed towards egg attendance in favor of nest defense and aggression towards the damselfish. We further hypothesized that the AVT antagonist would reduce aggressive nest defense while at the same time increase egg attendance, whereas the IT antagonist would have the entirely opposite profile, reducing egg attendance while increasing aggression. Such an observation would constitute strong evidence that the nonapeptides have opposing roles in the regulation of two distinct parental care behaviors, nurturing versus aggression. If true, the nonapeptides would be well-positioned for regulating shifts between parental care behaviors.

2. Methods

2.1. Animals and husbandry

Two species of teleost fishes were used in this study, the False Clown anemonefish (Amphiprion ocellaris) and the Three Spot Domino damselfish (Dascyllus trimaculatus). The False Clown anemonefish were 2nd and 3rd generation fish bred and raised in the laboratory from broodstock obtained via ORA (Ocean Reefs and Aquariums, Fort Pierce, FL), while the Three Spot Domino damselfish were obtained directly from the aquarium pet trade (Saltfin Aquatics, Champaign, IL). Domino damselfish naturally cohabit with A. ocellaris and will consume eggs if not aggressively forced away by parents (personal observation). All fish in this study were housed in 20-gallon tall aquariums with similar environmental conditions. Aquariums were integrated via a large central filtration system with a tank water volume turnover rate of 20 times per hour to ensure comparable water chemistry parameters across tanks and within the system. Environmental conditions were set to mimic the natural environment with a photoperiod of 12:12, the temperature set at 79 °F, pH between 8 and 8.4, and specific gravity of 1.026. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Prior to experimental manipulations, A. ocellaris were kept in pairs consisting of one dominant female and one subordinate male. Each tank also contained a terra-cotta pot (6-inch diameter) to provide a protective nest site and spawning substrate for fish to deposit their eggs. D. trimaculatus were kept in groups of 7–10 individuals in tanks containing multiple terra-cotta pots and PVC tubes for safe refuge. Fish were fed at...
least twice a day to satiety with a mix of New Life Spectrum Marine (Homestead, FL) pellets and Golden Pearls (Ogden, UT), with frozen adult brine shrimp and mysis shrimp supplemented approximately once per week.

A total of 10 experimental tanks were used in this study, each containing one male and one female A. ocellaris at least 3 years of age and reproductively active (spawn intervals of < 21 days). The mean body length (as measured from the tip of the nose to terminus of the caudal fin) for females was 7.24 cm (± 0.61 SD, range 6.3–8.0 cm), females had an average body mass of 8.56 g (± 2.1 SD, range 5.67–11.07 g). The males had a mean body length of 5.43 cm (± 0.43 SD, range 4.6–5.9 cm) and mass of 3.35 g (± 0.72 SD, range 2.21–4.45 g).

A total of 20 Damselfish were used as intruders and were introduced from their group tank to the experimental tanks with the A. ocellaris pairs for short intervals as described below in more detail. Damselfish were not sexed and had an average length of 47 mm (37–57 mm) and average weight of 3.34 g (1.4–5.4 g). All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

2.2. Experimental design

Parental behavior of male and female A. ocellaris was first quantified during an unmanipulated egg rearing period (N = 10). Pairs of A. ocellaris were continually monitored until a spawning event occurred. Photos of the nest were taken on the day the eggs were deposited, and again on the 6th day after egg deposition following video recordings. Video recordings were taken between 2:00–3:00 pm on the 4th, 5th, and 6th day after eggs were laid (Fig. 1). Days 4, 5, and 6 of the egg-rearing period were chosen for behavioral observation because these days have been previously shown to have high levels of parental care with lower individual variation across days compared to other time points during egg rearing. Additionally, these days were chosen for consistency with previously published works within our laboratory (DeAngelis et al., 2018; DeAngelis et al., 2017a; DeAngelis and Rhodes, 2016). Video recordings were conducted for 15-min, allowing time for fish to acclimate to the video camera being setup outside their tanks. While fish occasionally swim behind the terra-cotta pot, they do so only briefly, and are in view and observable almost in entirety. Videos were then scored by the same individual for a 5-minute quantification period using BORIS event-logging software (Friard and Gamba, 2016). The proportion of time spent in the nest (whole body within the terra-cotta pot), the number of nips (mouthing the eggs to keep them free of debris and fungus), and the number of fans (using fins to move oxygen rich water over the eggs) were recorded and quantified.

Subsequent to behavioral observations during the initial unmanipulated egg rearing period, parental care and aggressive egg defense were quantified during 3 additional spawning periods. Pairs of A. ocellaris were continually monitored for a spawning event as described above except, in each spawning sequence, 3 Domino damselfish were introduced into their tanks immediately before video recordings on behavioral observation days 4, 5, and 6 (Fig. 1). Damselfish were not individually identified, and were caught randomly from their home tanks, then released into A. ocellaris tanks each day. In addition to
measuring parental care behaviors as described above, the number of charges (attempt to attack the damselfish by full speed charge) were also recorded separately for both males and females. Damselfish were removed from the home tanks of A. ocellaris pairs immediately following video recordings each day to reduce unnecessary aggression, and potential for the A. ocellaris pair to acclimate to the damselfish intruders.

A. ocellaris males received one of three pharmacological treatments during three experimental spawning series. Males received intraperitoneal injections of either an AVT antagonist, IT antagonist, or vehicle saline control in a volume of 10 μl/g body weight. The order of treatments was counter balanced across tanks in order to control for any ordinal effects of pharmacological manipulations (Table 1). Males were caught from home tanks with a net and restrained by hand. Injections were then administered via a 100 μl syringe (BD syringe, 26 gauge, 5/8”). Fish were injected approximately 5 mm anterior to the urogenital orifice and immediately placed back into their home aquarium. Injections were given 30 min prior to video recordings and behavioral observations. This time window was chosen as it has been previously validated as enough time to allow fish to recover from the netted removal from their home tanks and injection stress, while still within the time frame when antagonists are presumed to be behaviorally active within the brain (DeAngelis et al., 2017b; O’Connell et al., 2012; Semsar et al., 2001). Males received the IT antagonist desGly-NH2-d(CH2)3[D-Tyr2,Thr4](OVT (a gift, courtesy of Dr. Maurice Manning) at a dose of 0.5 μg/g body weight. Importantly, this is a highly selective OT antagonist (Cho et al., 2000; Manning et al., 2012). This dose was chosen as we have previously demonstrated it significantly affects behaviors specific to parental care (DeAngelis et al., 2017a), and is also the minimal dose needed that is known to affect paternal behavior in the closely related cichlid fishes Amatitlania nigrofasciata and Neolamprologus pulcher (O’Connell et al., 2012; Reddon et al., 2014). The AVT (V1a) receptor antagonist d(CH2)3[Tyr(Me)2]AVP, Manning compound (Fisher Scientific, Waltham, MA, USA) was administered at a dose of 3.2 μg/g body weight. This dose was chosen as we have previously validated its effect on aggression (Yaeger et al., 2014) and parental care (DeAngelis et al., 2017a).

2.3. Statistical methods

Data were analyzed using R (version 3.5.1 ‘Feather Spray’) statistical software (Team, 2014). \( P < 0.05 \) was considered the threshold for statistical significance. Residual distributions from the statistical models were inspected visually for normality and also tested using a Shapiro-Wilks normality test with \( P > 0.10 \). During the initial observational spawning period, parental behavioral acts were averaged for each individual and for each sex across days 4, 5 and 6. Means were then compared between the sexes using a paired \( t \)-test.

In order to determine the effect of nest predator damselfish on the parental behavior of amonofish, a linear mixed effects model (LME, LME4 package, (Bates et al., 2014)) was performed. Two treatments were compared in this model, the initial observational spawning period and the saline treatment during damselfish intrusions. Total parental behaviors of both males and females as measured on days 4, 5, and 6, were modeled as a function of treatment and sex as fixed factors, with a treatment-by-sex interaction and individual as a random effect in order to account for repeated measures across treatments. Significance of the random effect was conducted using the ‘rand()’ function within the ‘lmer’ package within R, which compares the models with and without random effects creating a vector of Chi square statistics and corresponding \( P \) values for random factors (Kuznetsova et al., 2015). Effect sizes for this model were calculated as a correlation between the fitted and observed values and are reported as \( R^2 \). Post hoc pair wise comparisons were performed using Fisher’s protected Least Significant Difference (LSD) tests. Effect sizes for pairwise comparisons were calculated using the cohensD() function from the ‘lsr’ package (Navarro, 2013) in R.

To establish the effect of antagonist treatments on parental behaviors and time spent in the nest, an LME model was conducted within each sex independently as only males received antagonist treatments. Within males, total parental behaviors, the number of aggressive charges, and proportion of time spent in the nest were modeled independently as a function of antagonist treatments, with individual (unique identifier) entered as a random effect in order to account for repeated measures across antagonist conditions. Female behavior was modeled as a function of male behavior, as females did not receive antagonist injections. Total maternal behaviors, and proportion of time females spent in the nest were modeled as a function of male parental behaviors, and male aggression. Within each LME model, post hoc analyses were conducted using a Fisher protected LSD test at pair-wise comparison rate, given we had an a priori prediction that each antagonist treatment would differ from the saline control.

3. Results

3.1. Effect of nest predators on parental behaviors

During the initial spawning period, males displayed an average of 89.3 (± 8.71 SEM) total egg care behaviors (sum of nips and fans) and spent 81% of their time in the nest. Females exhibited an average of 129.0 (± 3.09 SEM) total egg care behaviors and spent only 29% of their time in the nest. Consistent with previous reports (DeAngelis et al., 2018; DeAngelis et al., 2017b; DeAngelis and Rhodes, 2016), males displayed significantly more total egg care behavioral acts \( [t_9 = 7.76, \text{Cohen’s } d = 3.27, P < 0.0001] \), and spent a significantly higher proportion of their time in the nest as compared to females \( [t_9 = 5.33, \text{Cohen’s } d = 2.05, P < 0.0001] \).

Following the introduction of damselfish, males and females defended the eggs with equal effort (Fig. 2; only data for saline vehicle treatment shown \( [t_9 = 0.94, \text{Cohen’s } d = 0.42, P = 0.36] \). In order to understand how the introduction of damselfish during the egg-rearing period affects parental care, comparisons of parental behaviors during the initial no treatment observational period, and the saline control treatment during damselfish introductions were analyzed. Results from the LME model indicate that the addition of damselfish into the tanks of A. ocellaris pairs during the egg-rearing period had a significant effect, reducing the amount of direct egg care behaviors \( [F_{1,103.4} = 71.33, R^2 = 0.656, P < 0.0001] \); the random effect was also significant \( [P < 0.001] \), indicating that individuals vary in their level of aggression. Furthermore, sex was also a significant factor \( [F_{1,103.4} = 86.17, R^2 = 0.51, P < 0.0001] \), and a significant interaction between sex and treatment was present \( [F_{1, 103.4} = 43.00, R^2 = 0.26, P < 0.0001] \), indicating that males shift their parental care strategy in response to the introduction of damselfish intrusions while females remain unchanged (Fig. 3). Post hoc comparisons within each sex show that males displayed 81% fewer parental behaviors when damselfish were present \( [\text{Cohen’s } d = 2.02, P < 0.001] \), while no differences in parental effort

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Table 1
Order of treatments for each experimental male.

<table>
<thead>
<tr>
<th>Tank</th>
<th>Spawn 1</th>
<th>Spawn 2</th>
<th>Spawn 3</th>
<th>Spawn 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>B13</td>
<td>None</td>
<td>IT</td>
<td>S</td>
<td>AVT</td>
</tr>
<tr>
<td>B23</td>
<td>None</td>
<td>AVT</td>
<td>S</td>
<td>IT</td>
</tr>
<tr>
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<td>S</td>
<td>IT</td>
<td>AVT</td>
</tr>
<tr>
<td>A24</td>
<td>None</td>
<td>S</td>
<td>IT</td>
<td>AVT</td>
</tr>
<tr>
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<td>AVT</td>
<td>IT</td>
<td>S</td>
</tr>
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<td>S</td>
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<td>IT</td>
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<tr>
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<td>IT</td>
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<td>S</td>
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<td>AVT</td>
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<td>IT</td>
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<tr>
<td>B12</td>
<td>None</td>
<td>AVT</td>
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<td>IT</td>
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\( R. \text{ DeAngelis, et al.} \) Hormones and Behavior 121 (2020) 104717
were signifi cant for females [Cohen’s d = 0.74, P = 0.185].

3.2. Effect of nonapeptide antagonists on aggressive defense of eggs

Results from the LME model showed that the antagonist treatments had an overall signifi cant effect on parental aggression [F2,71.25 = 14.17, R2 = 0.622, P < 0.001]. Additionally, and consistent with previous reports (DeAngelis et al., 2018; DeAngelis et al., 2017b), there was a signifi cant random effect of the individual (P < 0.0001), indicating that individuals differ in their aggressive behavioral displays independent of treatment. Post hoc pairwise comparisons showed that males were signifi cantly more aggressive during the IT antagonist treatment; males displayed 2-fold the amount of aggressive charges during the IT antagonist treatment (Cohen’s d = 0.52, P = 0.002, Fig. 4A) compared to the saline control. Furthermore, males were signifi cantly less aggressive during the AVT antagonist treatment compared to the saline control (Cohen’s d = 0.65, P = 0.032), displaying 3.5-fold fewer aggressive behavioral displays (Fig. 4A). This result shows that each antagonist treatment had a signifi cant and opposite effect on paternal aggression, with the IT antagonist treatment increasing aggression, and the AVT antagonist treatment decreasing aggression.

Within females, results from the LME showed that the aggressive behavior displayed by males was signifi cantly positively correlated with maternal aggression across treatments [F1,8.4 = 6.71, R2 = 0.52, P = 0.011]. A signifi cant random effect of individual was also present [P = 0.0001]. Females displayed 0.308 behavioral acts for every single behavioral act displayed by males.

3.3. Effect of nonapeptide antagonists on parental effort and proportion of time in nest

Overall, results from the LME indicate that the antagonist treatments had a signifi cant effect on male egg care effort (sum of nips and fans) [F2,72.11 = 7.29, R2 = 0.51, P = 0.0013]. Furthermore, and consistent with fi ndings on aggression, there was a signifi cant random effect due to individual variation (P < 0.0001). This effect indicates that individual males vary in their overall egg care eff ort across spawning periods. Post hoc analysis showed that only the IT antagonist treatment signifi cantly affected male egg care eff ort in comparison to the saline control (Cohen’s d = 0.53, P = 0.027). Individuals receiving the IT antagonist displayed only 58.8% the number of total egg care behaviors as saline controls (Fig. 4B), a result consistent with previous fi ndings showing that blockade of IT signaling reduces paternal care (DeAngelis et al., 2017b). While not signifi cant (Cohen’s d = 0.28, P = 0.11), there was a trend for an increase in eff ort during the AVT antagonist treatment compared to saline control. Males displayed 1.27-fold more parental behaviors during the AVT antagonist treatment compared to the saline control (Fig. 4B). These fi ndings are consistent with fi ndings on aggression, suggesting that the signaling pathways of AVT and IT infl uence paternal care in opposite ways (DeAngelis et al., 2017b).

Results from the LME model showed that the egg care behavior of males had no signifi cant eff ect on the egg care eff ort of females [F1,64.4 = 3.19, R2 = 0.25, P = 0.079]. Within the model, only the random eff ect was signifi cant (P = 0.04), indicating that individual females vary in their total eff ort across spawn periods.

Antagonist treatments had no signifi cant eff ect on the amount of time males spent in the nest [F2,72 = 2.34, P = 0.103]. Within the model, only the random eff ect was a signifi cant factor in the variation of time spent in the nest (P < 0.0001). Similarly, the antagonist
treatment given to males did not affect the proportion of time females spent in the nest \( \left( F_{2,72} = 2.34, R^2 = 0.59, P = 0.10 \right) \).

4. Discussion

The primary finding of this study is that the two aspects of parental care studied, direct caring of the eggs (nipping and fanning) and egg defense (aggressive attacks towards nest intruders), are regulated by IT and AVT in diametric ways. These data support the hypothesis that IT signaling is necessary for males to shift their attention away from nest defense and towards direct care for the eggs, while AVT signaling is necessary for doing the opposite, shifting from nurturing to aggression. Taken together, we speculate that AVT and IT signaling contribute competing information to the neural components which resolve trade-offs in parental care strategy.

Previously, without introducing nest predators, we showed that when given an AVT antagonist, males increase the number of egg care behaviors they perform (DeAngelis et al., 2017b). Given the known role of AVT signaling in aggression (DeAngelis et al., 2017b; Greenwood et al., 2008; Huffman et al., 2015; Ten Eyck and ul Haq, 2012; Yaeger et al., 2014), we hypothesized that this increase in care was a response of reduced vigilance, therefore allotting more parental effort to be directed specifically towards egg care. We reasoned that even if there were no nest predators around, cognitive attention could still have been devoted to vigilance, and if that attention was reduced by the AVT antagonist treatment, this would allow more attention to be directed towards egg care. Results from the present study, with the addition of potential nest predators, are indeed consistent with this hypothesis. The parental behaviors, nest defense and egg care, were reciprocally related in such a way that if one behavior was decreased by an antagonist treatment the other was increased. Taken together, our data suggest that IT and AVT play an important reciprocal role in mediating the appropriate behavioral output parents must deliver in the trade-off between nest defense and direct parental investment.

4.1. Parental behavioral responses to changes in predation pressure

The introduction of damselfish dramatically altered the parental behavior of males. Following the introduction of potential nest predators, males shifted their parental strategy, from direct egg care to aggressive defense of the nest. When intruders are present, an increase in aggressive defense of nest is required, whereas higher levels of direct parental care may promote reproductive success when intruders are absent. This shift in males from high levels of direct care to high levels of aggression is consistent with other species where the introduction of nest predators reduces provisioning of the young (Ghalambor and Martin, 2002; Lissåker and Kvarnemo, 2006).

In females, the direct level of parental care is not significantly affected by the addition of damselfish, however, this is likely due to the small effect size for females, which are already displaying a low number of parental care behavioral acts as compared to males. Sex specific responses to variability in the parental environment are not uncommon. In burying beetles, males increase their parental investment following removal of their female partner, while conversely, females do not change their investment following male removal (Rauter and Moore, 2004). The opposite sex specific patterns are true in orange tufted sunbirds; when the foraging efficiency of females was experimentally reduced, males failed to compensate to the extent females did in the opposite condition. Hence, our result that males respond with a significant reduction in direct parental investment compared to females is not uncommon and likely reflects the mating strategy in A. ocellaris in which the male is the primary provisioning of the eggs (Fig. 3).

While this current report, as well as previous studies within our lab show that males are the predominant care giver in terms of direct care of the eggs (i.e., nipping and fanning), here, we observed no sex difference in aggression towards intruders (Fig. 2). This suggests that females may play a previously underappreciated role in parental care in A. ocellaris, in the form of nest defense (DeAngelis et al., 2018; DeAngelis et al., 2017b; DeAngelis and Rhodes, 2016). Furthermore, there is a significant positive relationship between male aggression and female aggression across treatments, suggesting that males and females are coordinating their efforts in the aggressive defense of the nest.

4.2. AVT signaling is required for shifting attention towards nest defense

While the role of AVT in aggression has been extensively studied and has repeatedly been implicated as a positive facilitator of aggression, dominance, and vigilance, far fewer works have addressed the influence of AVT signaling during parental care, and thus far results have been conflicting. Our results suggest AVT signaling is required for increased aggressive nest defense at the expense of direct egg care. In a
study of prairie voles, data suggest that injections of AVP into the lateral septum increase male parental responsiveness, but the effects of AVP were dose and brain region specific (Wang et al., 1994). Similarly, in two species of sex-role reversed pipefishes, males incubating eggs in brooding pouches show increased levels of AVT compared to non-parental males, but vigilance and aggression were not measured (Ripley and Foran, 2010). Hence, we do not know the extent to which this increase in AVT is a response of direct parental care, or whether it may be tied to heightened vigilance and aggressive defense of eggs. Further examination of different rates of nest defense and egg caring in pipefishes is needed to disentangle the specific role of increased AVT in paternal behaviors and how it may relate to findings presented here.

Our study is in line with previous reports suggesting a functional role of AVT/AVP during parental aggression and the role of AVT in mediating trade-offs in parental behavior.

In the uniparental paternal Puerto Rican coqui frog, males display two types vocalizations, including an advertisement call to attract females to their territories, and an aggressive call which is used both to defend territories from conspecics, and also to defend their offspring from predators during paternal care (Hayes, 2009; Stewart and Rand, 1991; Townsend et al., 1984; Wells, 1977). In a study by Ten Eyck and ul Haq, 2012 (Ten Eyck and ul Haq, 2012), the authors found that exogenous administration of AVT to actively parenting males increased the number of aggressive calls and also elicited more erect postures and increased behavioral activity. The number of advertisement calls also increased, but at a lower rate than did aggressive calls. Another recent study in a different tropical frog (Ranitomeya imitator), a biparental species, both males and females reduced the amount of intensive care (climbing and moistening of the eggs) displays following AVT injections without changing the amount of time spent with clutches. Furthermore, the authors comment that AVT injections seemed to increase the amount of general parental behaviors and highlight the possibility that AVT signaling engenders protective and inspection behavior, while intensive care behaviors are likely modulated by other neuroendocrine processes (Schulte and Summers, 2017). Taken together, these studies (along with our current report) demonstrate the regulatory complexity of distinct behavioral acts under the umbrella of parental care. More specifically, they suggest AVT signaling increases the aggressive defense of offspring at the cost of reduced caring of eggs. It would be interesting to see how direct administration of IT and AVT in the brain of parenting anemonefish affects parental behavior. This would further elucidate whether IT and AVT are involved in egg care vs. egg defense in this species.

4.3. IT signaling is required for shifting attention towards egg care

Our findings indicate that IT signaling is critical for high levels of direct parental care (nipping and fanning of the eggs). Males injected with an IT antagonist displayed a reduction of direct egg care behaviors. This was an expected result, given the extensive literature across several vertebrate lineages which consistently suggest that OT/IT signaling is a critical component of offspring care independent of which sex is the primary caregiver (DeAngelis et al., 2017b; Gubernick et al., 1995; Keverne and Curley, 2004; O’Connell et al., 2012; Olazabal and Young, 2006; Ragnauth et al., 2005; Roland and O’Connell, 2015; Saito and Nakamura, 2011). Furthermore, the behavior-reducing effects of the IT antagonist are at least somewhat specific to egg care. A previous study found no impact of the same dose of the IT antagonist on dyadic aggressive interactions between males (DeAngelis et al., 2017b). Additionally, the IT antagonist treatment had no effect on the amount of time spent in the nest. Hence, this reduction in egg care was caused by a reduced rate of parental behavior. When individuals were not parenting, or chasing damselfish, they display an up and down bobbing motion typical of anemonefish. Furthermore, in the present study, the IT antagonist increased (rather than decreased) aggression towards intruders, providing further support that the effect of the IT antagonist in reducing parental care behavior is specific to egg care. We speculate that the increase in nest defense comes from the release of attention towards egg care (which is the main response of the IT antagonist), hence allowing additional attention to be devoted to nest defense. We made a similar argument for reciprocal effect of the AVT antagonist on nest defense and egg care above.

In accordance with previously reported opposing effects of non-apeptide antagonists on direct parental care (DeAngelis et al., 2017b), here, we also see the opposite behavioral effects of IT and AVT receptor antagonists on nest defense aggression. To the best of our knowledge this is the first study to directly examine effects of an OT/IT antagonist on paternal aggression. In a study investigating both nest defense and parental care, conducted in the Three-Spined stickleback, males were placed in one of three conditions: males without nests, males without nests which were exposed to a mirror (to incite defensive territorial aggression), and males with nests and also exposed to a mirror. Of these three groups, only males with nests which were also exposed to a mirror displayed elevated levels of whole brain IT. In the stickleback model, nesting males display multiple behaviors in close temporal proximity, including nest building, parental care, courtship, and territory defense. Given the diverse role of IT in social behaviors, it is difficult to disentangle which specific behaviors involved IT. Nevertheless, in light of the fact that IT was not elevated in the mirror only condition, together with our data which suggest IT facilitates egg care at the expense of egg defense, it is likely IT increased in the sticklebacks to facilitate egg care specifically.

Thus far, studies exploring the role of OT/IT in parental aggression have been conducted primarily in female laboratory rodents and have provided conflicting results. While the majority of studies conducted suggest that OT signaling reduces maternal aggression, others suggest the opposite pattern, which may be confounded by the highly dependent nature of OT signaling on genotype, sex, social status, and social context (Bosch, 2013; Shamay-Tsoory and Abu-Akel, 2016). Following infusion of an OT antagonist into the central nucleus of the amygdala (CNA), maternal rats display increased aggression, attacking intruders significantly more than controls. (Lubin et al., 2003). These results are consistent with our current findings, and also support the CNA as an important brain region in the regulation of anxiety and aggression in defense of offspring. Another study of maternal rats reported that infusion of an OT antagonist into the prefrontal cortex increased anxiety like behaviors in postpartum, but not virgin females. Furthermore, blockade of OT postpartum impaired maternal care behaviors while increasing maternal aggression. Specifically OT blockade reduced latency for dams to attack intruders and increased their number of attacks (Sabhi et al., 2014). Lastly, in Syrian hamsters, OT injections directly in the medial preoptic anterior hypothalamic continuum reduced aggressive displays in females during antagonistic encounters with conspecifics, while treatment with an OT antagonist increased aggression (Harmon et al., 2002). These results are consistent with our present findings, and also highlight the importance of reproductive state (i.e. whether parenting or not) in the role of OT in aggression. The medial and basolateral amygdala are important brain regions for fear, learning and parental care, and several studies have demonstrated that AVP and OT regulate circuits within the amygdala in opposite ways. AVP increases the probability that basolateral projections will excite neurons within the CeA, while OT displays the opposite pattern, reducing the same excitatory projections (Campbell-Smith et al., 2015; Huber et al., 2005; Lubin et al., 2003; Numan et al., 2010). This suggest that OT may be important in reducing anxiety and fear like behavioral responses, data which is consistent with our present findings where blockade of IT increased aggression, presumably a fear induced behavior. Taken together, these data suggest a potential mechanism for how AVT/AVP and OT/IT signaling might function to mediate allocation to different and potentially competing parental behaviors, specifically between nest defense and direct egg care by altering the
5. Conclusion

Individuals must incorporate complex information from their current environment and continually modify their behavioral output in order to maximize fitness. Results from this study suggest that the nonapeptides play a crucial, reciprocal role in mediating shifts between parental care behaviors of nest defense versus nurturing of the eggs depending on the presence of potential nest predators. Blockade of IT reduced direct egg care while at the same time increased aggressive charges towards the intruders. Conversely, blockade of AVT reduced aggressive displays towards intruders while tending to increase direct egg care behaviors. Taken together with our previously published study (DeAngelis et al., 2017b), we conclude that AVT and OT play distinct roles in aggressive defense of eggs, and direct parental investment (nipping and fanning of the eggs), and function to mediate the trade-offs in parental displays in response to social perturbations.

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