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Opposite effects of nonapeptide antagonists on paternal behavior in the teleost fish *Amphiprion ocellaris*

Ross DeAngelis [a,b,*], Joseph Gogola [b], Logan Dodd [b], Justin S. Rhodes [a,b]

[a] Program for Ecology, Evolution and Conservation Biology, University of Illinois at Urbana, Champaign, 405 N. Mathews Ave, Urbana, IL 61801, USA
[b] Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana, Champaign, 405 N. Mathews Ave, Urbana, IL 61801, USA

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ABSTRACT

The nonapeptides isotocin (IT) and arginine vasotocin (AVT), along with their mammalian homologs oxytocin and arginine vasopressin, are well known regulators of social behaviors across vertebrate taxa. However, little is known about their involvement in paternal care. Here, we measured the effect of an IT and an AVT V1a receptor antagonist on paternal behaviors in the primarily paternal teleost *Amphiprion ocellaris*. We also measured the effect of the IT receptor antagonist on aggression in dyadic contests between two non-reproductive fish to assess specificity of the effect on paternal behaviors. Individual differences in levels of paternal behaviors (nips, fanning the eggs, and proportion of the time in the nest) were consistent across spawning cycles when no treatments were administered. The IT receptor antagonist severely reduced paternal behaviors but had no effect on aggression, whereas the AVT V1a receptor antagonist increased paternal behaviors. These results support the idea that IT signaling is crucial for the expression of paternal behavior in *A. ocellaris*. Based on a previous study showing that the AVT V1a antagonist decreases aggression in dyadic contests, we hypothesize that the antagonist enhances paternal behavior indirectly by reducing vigilance and aggression, thereby alleviating effort directed towards other competing behaviors and allowing for the increased expression of paternal behaviors.

1. Introduction

Parental care is a costly investment on the part of the caregiver as it presents a trade-off between current parental investment and opportunities for future reproductive events (Clutton-Brock, 1991; Trivers, 1974). Often, this trade-off is different for each sex. Male care is less common, as males produce a large number of metabolically inexpensive gametes, and generally have higher reproductive success siring as many offspring as possible (Kokko and Jennions, 2012; Trivers, 1972). Thus, paternal care is predicted to occur only when the cost of desertion is high, or future reproductive opportunities are low (Gross and Sargent, 1985). Conversely, females produce fewer gametes that are larger in size, and generally have higher reproductive success when effort is directed towards egg survival (Gross, 2005; Trivers, 1972). High rates of maternal care has consequently led to a bevy of studies on the evolution and underlying neural regulation of female parental care. However, unlike female parental care, there have been relatively few studies examining mechanisms underlying male parental care, despite the fact that in many species exhibiting male parental care, paternal effort is of equal importance to maternal care, and in some species even more important than female care for offspring survival (Kleszczyńska et al., 2012; Ripley and Foran, 2010; Rodgers et al., 2006; Trainor and Marler, 2002).

While paternal care is relatively uncommon, teleost fishes are a unique group among vertebrates in which male parental care is the predominant parental care strategy (Baylis, 1981; Magee et al., 2006; O'Connor et al., 2009; Pradhan et al., 2014). Recent work has presented evidence of a highly evolutionarily conserved social decision-making network. More specifically, the brain regions, neuropeptides, and hormones involved in the regulation of social behaviors share similar pathways across vertebrate taxa (O'Connell and Hofmann, 2011). Thus, teleosts present interesting opportunities to gain insight into the regulation of vertebrate parental care (Amundsen, 2003; Gross and Sargent, 1985; Ridley, 1978). To date, the few species of teleosts where paternal care has been explored present confounding results, which may in be in part due to the often simultaneously occurring social displays such as courtship and territory defense (Kleszczyńska et al., 2012; O'Connell et al., 2012). Hence, the high homology of the underlying circuitry involved in the regulation of social decisions allows insights to be gained broadly about fathering by studying species where high levels of paternal care are exhibited and in which care can be isolated form other confounding social behaviors (Amundsen, 2003; Goodson, 2005; O'Connell et al., 2012).

* Corresponding author at: Beckman Institute, 405 N. Mathews Ave., Urbana, IL 61801, USA.
E-mail address: deangelr@illinois.edu (R. DeAngelis).

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The anemonefish, *Amphiprion ocellaris* is a predominantly paternal care species in which male care is critical for offspring survival (DeAngelis and Rhodes, 2016). Their obligate symbiosis with protective host sea anemones and highly monogamous lifestyle has removed opportunities of finding additional mates. (Godwin, 2009; Mitchell and Dill, 2005). Hence, the substantial effort of parental care does not come at the expense of seeking additional reproductive partners, as in other species where additional mating opportunities are more pervasive. This leads to an increased fitness by directing effort towards current brood survival and enables research to be directed specifically at paternal care in isolation of other confounding social displays. In addition, *A. ocellaris* is particularly well suited for laboratory studies on paternal care due to their small home ranges and adaptability to aquarium conditions (Iwata et al., 2008). They also have a short generation time (1 year) and spawn readily in captivity. Parental behaviors are easily observed and quantified, and sex differences in parental care have already been established. Males are the predominant caretakers, and spend the majority of their lives caring for eggs (DeAngelis and Rhodes, 2016).

As an initial foray into the regulation of the high levels of paternal care displayed by *A. ocellaris*, the vasopressin and oxytocin systems were logical candidates for exploration. The vasopressin and oxytocin systems are well situated for functioning in parental care as cell bodies containing these neuropeptides reside in the preoptic area of the hypothalamus, an area of the brain well known for regulating reproductive behaviors (Insel and Young, 2000; Kline et al., 2011). The cell bodies and dendrites heavily express sex steroid receptors, and project axons all over the brain, the terminals of which release the neuropeptides onto multiple neuron types (Foran and Bass, 1999). Receptors for these neurochemicals are known to be expressed in crucial brain areas that comprise the social decision-making network such as ventral midbrain, basal ganglia, and hippocampus (O'Connell and Hofmann, 2011). Nonapeptide cells in the brain receive information from the gonads via the blood and relay that information to the rest of the brain for making social decisions related to reproduction (Maruska and Fernald, 2011). While a relationship between these highly conserved neuropeptides and maternal care and other social behaviors has been identified, the extent to which these nonapeptides play a role in paternal care remains unclear.

The neuropeptide oxytocin (OT) has been well studied for its role in female parental care; its release at parturition is a catalyst for an array of behavioral and physiological changes critical for offspring survival (Bartz et al., 2010; Francis et al., 2000). While less understood in males, it has been suggested that OT plays a similar role in paternal behavior. In humans, OT rises in males following contact with an infant (Feldman et al., 2010); in the monogamous California mouse *Peromyscus californicus*, OT levels are higher in expectant fathers (Gubernick et al., 1995); and in teleost *Amatitlania nigrofasciata* blockade of the IT receptor reduced paternal effort (O'Connell et al., 2012). These results support a conserved function of OT in the promotion of parental care.

Like OT, the neuropeptide AVP/AVT has a well-documented role in the regulation of social behaviors across a wide array of vertebrates, and has been suggested to be more important in regulating male social behaviors (Insel and Young, 2000). In teleosts, AVT is broadly implicated in behaviors leading to reproduction, but its specific function varies depending on species and social status (Foran and Bass, 1999; Insel and Young, 2000; Kleszczyńska et al., 2012). More specifically, AVT has been implicated in the regulation of dominance, aggression, and courtship (Huffman et al., 2015; Semsar et al., 2001; Yaeger et al., 2014). AVP/AVT is clearly important in a variety of male social behaviors, but surprisingly few studies have addressed its role in paternal care, and therefore how AVT signaling functions to promote or inhibit male parental care remains unclear.

The goal of this study was to determine the extent to which AVT and IT signaling play a role in the modulation of paternal care in a species where high paternal effort can be isolated in the absence of other confounding co-occurring social behaviors often exhibited in other species. In this study we hypothesize that IT signaling is critical for high levels of paternal care, and thus, blockade of IT signaling will reduce total parental effort. Given that blockade of AVT V1a receptors reduced aggression in *A. ocellaris*, and the diversity of roles reported for AVT in the regulation of multiple different competing social behaviors, we were not certain how blockade of V1a receptors would affect paternal care.

2. Materials and methods

2.1. Animals and husbandry

A. ocellaris, bred in the laboratory from a female obtained from ORA (Oceans Reefs and Aquariums, Fort Pierce, FL), and a wild caught male (location unknown; obtained through the pet trade) were used. Tank conditions for all individuals were set to mimic the natural environment, with a temperature of 79 °F, photoperiod of 12:12 (lights on at 7:00 am and off at 7:00 pm), pH of 8.2 and specific gravity of 1.026. Individuals were housed in groups of 2 or 3 in 20-gallon aquariums, and allowed over a year for consistent spawning (spawn period of <20 days) prior to the onset of behavioral observations or experimental manipulations. Each tank contained one clay pot (4-inch diameter) to serve as the nest site where the fish deposit their eggs. Lengths and weights were taken one week prior to the start of the experiment and then again at the conclusion of the study. In experiment 1, mean body weight and standard length were 2.97 g (range 1.60–5.70 g) and 53 mm (45–70 mm) for males and 7.38 g (4.60–9.60 g) and 70 mm (46–96 mm) for females. In experiment 2, mean body mass and standard length of the males were 2.60 g (range 1.88–3.44 g) and 45 mm (38–53 mm). Fish used in experiment 1 were approximately 24 months of age, while fish used in experiment 2 were approximately 18 months of age. All fish in experiment 1 had previously been observed caring for a batch of fertilized eggs and were established as reproductively mature. The fish in experiment 2 were non-reproductive males taken from a holding tank, which contained >20 fish (i.e., no reproductive pairs could be established). Adequate measures were taken to ensure minimal pain and discomfort for all animals used in experimental procedures. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

2.2. Experiment 1: Effects of AVT and IT antagonists on paternal behavior

2.2.1. Baseline measures of parental behavior, sex differences and consistency across breeding cycles

Aquariums that contained reproductive pairs ($N = 8$) were recorded daily for a 10-minute behavioral observation period (between 2:00–3:00 pm daily) over the entire spawn cycle. Behavioral analysis for spawn period 1 (SP1) began on the day the eggs were laid (day 0) and ended when eggs hatched into the larval phases (7–9 days). Parental behaviors were scored using JWatcher behavioral event recording software (Blumstein and Daniel, 2007). The amount of time spent in the nest, as well as the total number of nips and fans were quantified for the reproductive male and female in each aquarium. Nips are defined as mouthing the eggs to keep them clean of debris and fungus, while fanning is the process of using the pectoral and caudal fins to aerate the eggs. Total numbers of parental behaviors (the sum of nips and fans) were analyzed. This procedure was repeated again during spawn period 2 (SP2) to test the extent to which individual variation in behavior was consistent across spawn periods, before starting the pharmacological manipulations.

2.3. Pharmacological manipulations

Only males received the pharmacological manipulations. Following the analysis above, during the subsequent spawn period 3 (SP3),

males were restrained by hand and received an intraperitoneal (i.p.) injection (BD syringe, 3 mm, 26 gauge) of 0.9% saline (control) at 10 μl/g body weight on days 4, 5, and 6 after the eggs were laid ($N = 8$). These days were chosen based on when the greatest rise in parental effort was observed during SP1 and SP2 (see Results section). Immediately after the injection, fish were placed back into their home aquarium. Parental behaviors were recorded as described above, 30-minutes after the injection for a 15-minute duration. Behavioral analysis started 30-minutes post injection to allow fish enough time to recover from handling stress but still within the time window when the antagonists are expected to be at pharmacologically significant levels in the brain (O'Connell et al., 2012; Semsar et al., 2001).

During spawn period 4 (SP4), males were given an i.p. injection of an IT receptor antagonist, again on days 4, 5, and 6, and behavior scored a similar way ($N = 8$). The oxytocin receptor antagonist desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT (a gift courtesy of Dr. Maurice Manning) was administered at a dose of of 0.5 μg/g body weight. This dose was chosen, as it is 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).

Individuals were then allowed 3 spawn periods (approximately 28 days for most pairs) to recover from any effects caused by the IT antagonist injections. Following this period, fish were then given the AVP (V1a) receptor antagonist d$(CH_2)_5$[Tyr(Me)^2]AVP, Manning compound (Fisher Scientific, Waltham, MA, USA) again on days 4, 5, and 6, and behavior scored as described above ($N = 7$). A dose of 3.2 μg/g body weight at a volume of 10 μl/g body weight was used as this dose is known to block V1a receptors in the brain of teleosts, with almost no antidiuretic activity (Guillon et al., 2004). Moreover, this dose was previously found to block aggressive behavior in inter-male contests between *A. ocellaris* (Yaeger et al., 2014). Antagonist treatments were given serially rather than counterbalanced, a limitation addressed further in the discussion.

2.4. Experiment 2: Effect of IT antagonist on paired aggression trials

To determine the extent to which the IT antagonist alters aggressive and/or submissive behaviors, we used a previously validated paired aggression test which revealed positive results for the AVT antagonist (Yaeger et al., 2014). Two non-reproductive males were removed from their home aquariums where they were group housed with approximately 30 individuals, and each was placed into a separate 5-gallon bucket to await their injection. Fish were selected from separate home tanks so that in each trial, individuals had never interacted before and there were no established dominance relationships among individuals tested. Fish were sized matched to within 0.2 g body weight, and 4 mm in length. One fish was randomly assigned to receive a saline injection of 10 μl/g body weight ($N = 18$), and the other an IT antagonist injection at a dose of 0.5 μg/g body weight ($N = 18$), as described above. After the fish were given their injections, they were placed back into their 5-gallon buckets for a 30-min holding period. Following this 30-minute period, the two size matched fish were simultaneously placed into a 15-gallon aquarium and video recorded for 10 min. Aggressive, affiliative and submissive behaviors were scored. Bites and charges (rapid approach with an outstretched operculum) were scored as aggressive. Approaches (non-aggressive encounter), concurrent swimming (swimming within a body length), and touching were scored as affiliative, while quivering (rapid shaking, a submissive behavior in *A. ocellaris)* and fleeing were scored as submissive.

2.5. Statistical methods

Data were analyzed using R (version 3.3.0 'Supposedly Educational') statistical software (Team, 2013). $P < 0.05$ was considered statistically significant. To establish consistency of individual differences in paternal behavior across the two baseline spawn cycles, data were analyzed using a linear mixed model (Pinheiro et al., 2014). Total parental behaviors and duration in the nest across all the days were analyzed as a function of spawn period (SP1 and SP2) with each aquarium or pair (as the unique identifier) entered as a random effect in the model to account for the repeated measures between spawn cycles. Significance of the random effect was evaluated using a chi-square log-likelihood test comparing the model with versus without the random effect. Lack of an effect of spawn cycle and a significant amount of variation explained by the aquarium random effect was taken to indicate that the behavior was consistent across spawn periods with repeatable differences in behavior represented by the different pairs. Effect sizes were calculated from the variance components estimated by the mixed models and are indicated as R^{2*}.

Sex differences in parental behaviors across the breeding cycle were analyzed using a linear mixed effects model with day entered as the within-subjects factor, and sex as a between subjects factor. The behaviors were averaged across the SP1 and SP2 to produce one value per day per sex. Behavior of each sex was also analyzed separately using a simpler mixed model with only day entered as the within-subjects factor. Post-hoc, pair-wise differences between means were analyzed using Tukey tests. Effect sizes for model parameters were given as eta^2. To account for the repeated structure, data were first were analyzed with a one-way ANOVA with tank as a fixed effect (tank was entered as a random effect in the linear model described above). Residuals from this model were then analyzed by a two-way ANOVA and eta^2 extracted. The effect sizes for paired comparisons were given as Cohen's D.

The effect of the IT and AVT antagonists on paternal behaviors were analyzed as follows. First, the behaviors (total parental behaviors and duration in the nest) were averaged for each individual on the days fish received injections (days 4, 5, and 6 after the spawning event). These values were then analyzed using a linear mixed model with treatment entered as a fixed effect (4 levels: non-treated control, the saline injection, the IT antagonist, and AVT antagonist) and subject entered as a random effect to account for the repeated measures across the three treatment levels. The non-treated control was the average of both SP1 and SP2 for days 4, 5, and 6 to produce values (for parental behaviors and duration in nest) that were comparable to the saline and neuropeptide receptor antagonist values. Post-hoc, pair-wise differences between means were analyzed using Tukey tests (Hothorn et al., 2008). Effect sizes were calculated as described above.

For the paired aggression trials, data were not normally distributed and were analyzed using a paired non-parametric Mann-Whitney *U* test. Total number of aggressive behaviors and submissive behaviors were separately compared, within subjects, between the IT-antagonist treatment relative to the saline control.

3. Results

3.1. Experiment 1

3.1.1. Baseline measures of parental behavior, sex differences and consistency across breeding cycles

Levels of paternal care and duration in the nest were not significantly different between spawn periods (Fig. 1). However, certain males displayed elevated levels of paternal effort (Fig. 1A) and duration in the nest (Fig. 1B) compared to others, which was repeated across both spawn periods. This was indicated by a significant random effect of individual (for total parental behaviors, $R^{2*} = 0.69$, $P < 0.001$, and proportion time in nest, $R^{2*} = 0.76$, $P < 0.001$) in the linear mixed models, but no effect of spawn period.

The total numbers of parental behaviors (Fig. 2A) and duration in the nest (Fig. 2B) were significantly higher for males than females. This was indicated by a significant effect of sex in the linear model (total parental effort, eta$^2 = 0.51$, $F_{1,14} = 46.2$, $P < 0.001$; proportion time in nest, eta$^2 = 0.65$, $F_{1,14} = 65.9$, $P < 0.001$). In both males and females, parental effort and duration in the nest escalated as the days progressed, with the

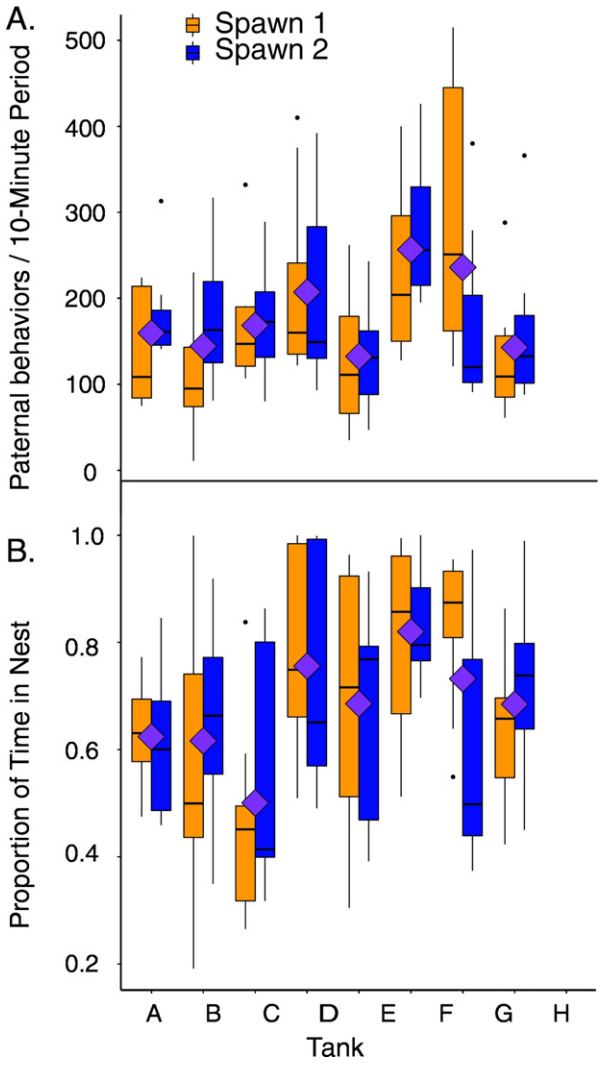

Fig. 1. Consistency of baseline levels of paternal behaviors. A) Box plot of total number of paternal behaviors (sum of nips and fans) displayed by 8 different males over two adjacent spawn periods (SP1 and SP2). B) Consistency of proportion of time spent in the nest. Significant differences were detected between males, but not between spawn periods. The line within the bars represents the mean. The purple diamonds between the adjacent bars represents the mean of SP1 and SP2 values.

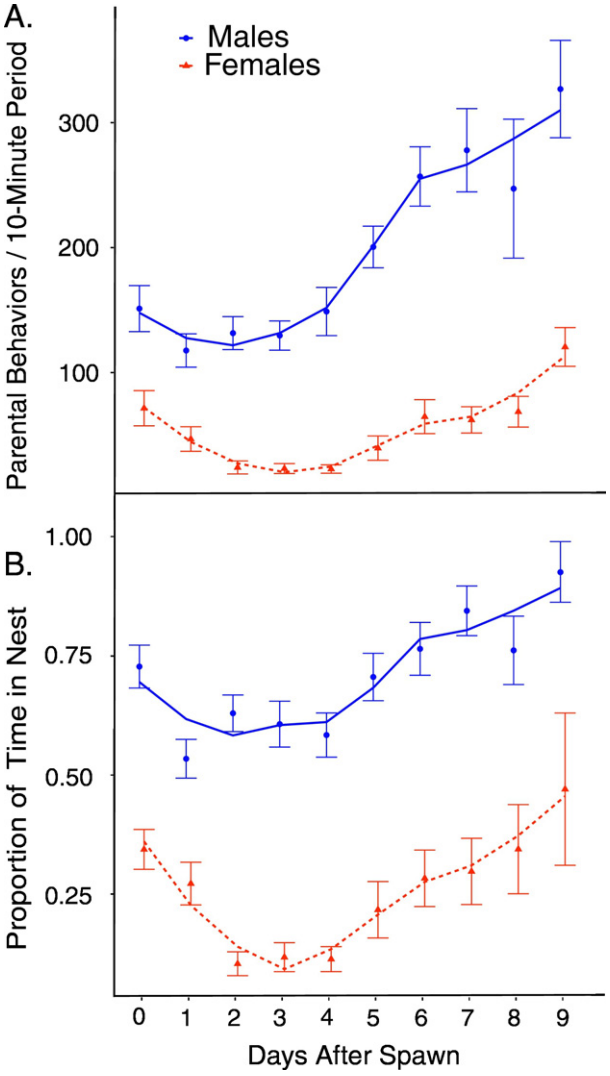

Fig. 2. Sex differences and patterns of parental behaviors across the spawning cycle. A) Average total number of parental behaviors (averaged across SP1 and SP2) are plotted per day separately for each sex (males in blue and females in red). B) Proportion of time in the nest is plotted. Loess curve and standard error bars are shown.

highest levels in the latter half of the breeding cycle, however the pattern was slightly different between the sexes (Fig. 2). This was indicated by a significant effect of day (total parental effort, $eta^2 = 0.13$, $F_{9106} = 7.7$, $P < 0.001$; proportion of time in nest, $eta^2 = 0.13$, $F_{9106} = 9.5$, $P < 0.001$) and a significant interaction between day and sex for total effort ($eta^2 = 0.07$, $F_{9106} = 3.8$, $P < 0.001$). For proportion of time spent in the nest, there was only a trend for an interaction between day and sex (eta = 0.03, $F_{9106} = 1.8$, $P = 0.084$). No other main effects or interactions were significant.

Considering males alone, parental effort escalated during the spawning period (Fig. 2A, $eta^2 = 0.49$, $F_{9,53} = 6.0$, $P < 0.001$), where it was lowest on day 1 and highest on day 8, with the most dramatic increases from days 4 to 5 and 5 to 6. Total parental effort was higher on days 6–8 compared to days 0–4 (all Cohen's D > 1.4, $P < 0.05$). Days 4–6 were targeted for pharmacological manipulations in subsequent spawn periods (see below) because this was the period of greatest escalation of paternal behavior. Duration spent in the nest also escalated as the eggs matured (Fig. 2B, $eta^2 = 0.48$, $F_{9,53} = 5.8$, $P < 0.001$), but the time-course was slightly different compared to females. Duration in the nest was high on day 0, and then lowered during days 1–4 before rising again on days 5–8. The largest increases in duration spent in the nest were from days 4 to 5, and from days 6 to 7. Proportion time spent in the nest was significantly higher on days 6–8, than 1–4 (all Cohen's D > 1.10, $P < 0.05$).

Considering females alone, parental effort ($eta^2 = 0.40$, $F_{9,53} = 4.1$, $P < 0.001$) and proportion of time in nest also escalated as the spawning period progressed ($eta^2 = 0.46$, $F_{9,53} = 5.4$, $P < 0.001$). Both maternal effort and time in the nest started high the day the eggs were laid and the following day after, and then lowered on days 2, 3 and 4, until escalating on days 5 through 9. Total parental effort and proportion time in the nest were lower on days 2, 3 and 4 than the other days (all Cohen's D > 0.85, all $P < 0.05$).

3.2. Effect of Nonapeptide Antagonists on Paternal Care and Duration in Nest

3.2.1. Paternal care

The repeated measures ANOVA indicated significant differences between the treatments for total number of paternal behaviors ($eta^2 = 0.57$, $F_{3,20} = 27.8$, $P < 0.001$; Fig. 3A). Post-hoc analyses of pair-wise

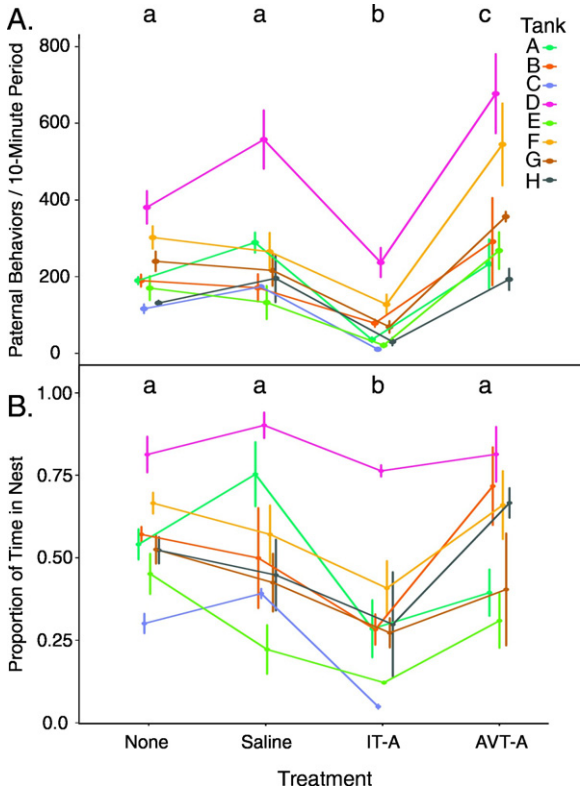

Fig. 3. Effects of IT and AVT V1a receptor antagonists on paternal behaviors. A) Average total number of paternal behaviors ± SE plotted for no-treatment (average of SP1 and SP2), in response to saline injection, IT antagonist, and AVT antagonist. The lines connecting the means correspond to the 8 individual males that were tested. The IT receptor antagonist significantly reduced paternal behaviors whereas the AVT V1a receptor antagonist significantly increased paternal behaviors. B) Proportion of time in the nest is shown. The IT antagonist reduced proportion time in nest relative to the other groups. Different letters indicate means are significantly different from each other by Tukey posthoc test at $p < 0.05$ level.

differences between means indicated that all pair-wise comparisons were significantly different from each other except saline and no-treatment controls (Cohen's D = 0.45, $P = 0.64$). The IT antagonist decreased total number of paternal behaviors, whereas the AVT antagonist increased paternal behaviors relative to both the saline (IT-Antagonist, Cohen's D = 2.28, $P < 0.001$, AVT-Antagonist, Cohen's D = 0.97, $P = 0.0038$) and no-treatment controls (IT-Antagonist, Cohen's D = 5.09, $P < 0.0001$, AVT-Antagonist, Cohen's D = 1.44, $P < 0.001$). The IT and AVT groups also differed from each other (Cohen's D = 2.58, $P < 0.001$).

3.3. Duration in the nest

Duration of time spent in the nest was positively correlated with total paternal behaviors across treatments ($F_{1,90} = 108.4$, $P < 0.001$, $R^2 = 0.55$). If a father in the nest was not actively caring for the eggs, it was simply swimming in one place in the nest next to the eggs and not otherwise engaging in any other behaviors.

The repeated measures ANOVA indicated significant differences between the treatments for duration spent in the nest ($eta^2 = 0.61$, $F_{3,81} = 13.57$., $P < 0.0001$; Fig. 3B). Posthoc analyses of pair-wise differences between means indicated that only the IT antagonist differed from the other groups. The IT antagonist significantly reduced duration in the nest relative to the saline (Cohen's D = 1.6, $p = 0.001$), no-treatment control (Cohen's D = 2.7, $P < 0.001$), and AVT antagonist (Cohen's D = 1.6, $P < 0.001$). No other pair-wise differences were significant.

3.4. Experiment 2

3.4.1. Effect of IT antagonist on aggression

The IT antagonist had no influence on aggressive or submissive displays compared to saline controls. Individuals treated with the IT receptor antagonist displayed an average of 79.4 (±20.54) aggressive acts, while saline treated animals displayed an average of 133.4 (±32.66) aggressive acts ($P = 0.083$). Individuals treated with the IT receptor antagonist displayed an average of 6.5 (±2.26) submissive behavioral acts while those treated with saline control displayed an average of 10.5 (±4.53) submissive acts. Displays of affiliative behaviors were extremely are, and due to low occurrence, were not statistically analyzed (data not shown).

4. Discussion

While the literature has established that both AVT/AVP and IT/OT are important neuromodulators of social behavior in vertebrates (Foran and Bass, 1999; Goodson, 2008; Goodson and Bass, 2001; Insel and Young, 2000), few studies have addressed the role of these neuropeptides in species that display predominantly male parental care where paternal care can be isolated form other co-occurring behaviors. The majority of the research has focused on female parental care where IT/OT signaling has proven critical for offspring survival (Bales and Carter, 2003; Francis et al., 2000; Olazabal and Young, 2006; Strathearn et al., 2009). Because of the diverse roles that these nonapeptides display in a species and ecological-context specific manner, it is important to explore their role in a primarily paternal care species. This work extends the literature by establishing that IT signaling is critical for paternal care in *A. ocellaris*. As opposed to IT/OT, the literature on AVT and parental effort is less consistent and more species specific (Foran and Bass, 1999; Kleszczyńska et al., 2012; Semsar et al., 2001). Here, these data add to the literature by showing that blockade of AVT signaling increases paternal care in a primarily paternal care species (Fig. 3). This is an intriguing result as males of this species already display incredibly high amounts of paternal behavior. The literature has established that in many teleost species, including *A. ocellaris*, AVT signaling is important in the regulation of dominance, aggression, and nest defense (Greenwood et al., 2008; Kleszczyńska et al., 2012; Semsar et al., 2001; Yaeger et al., 2014). We speculate that in *A. ocellaris*, blockade of AVT may increase paternal behavior by reducing attention towards vigilance and nest defense, thereby releasing attentional resources and effort to be directed towards parental care. However, this hypothesis was not directly tested here, and would have to be tested empirically before it can be confirmed. Taken together, these results establish the importance of both IT and AVT signaling as oppositely regulating paternal care in *A. ocellaris*.

4.1. IT and Paternal Care

This study demonstrates that IT signaling is critical for high levels of parental effort in a species that exhibits predominantly paternal care in isolation from other simultaneously occurring social behaviors. There was ample reason to suspect that the role of IT signaling could be different in *A. ocellaris* than in other species studied. In the uniparental three-spined stickleback, in which males are the sole caretakers of the eggs, IT does not appear to stimulate paternal behavior. Whole-brain IT levels, measured via high-performance liquid chromatography were highest during courtship and then lowered during parental care. IT was also higher in subordinate males displaying nuptial coloration compared to those not vying for a dominance position and reproductive opportunities (Kleszczyńska et al., 2012). These data suggest that IT is important for territory defense, and social status, but not necessarily paternal effort. However, in sticklebacks, males build nests where they actively defend a territory, court females, and care for broods. In this species males display many different behaviors simultaneously, making it difficult to

decouple the underlying neurobiological mechanisms specific to each behavioral phenotype.

In the majority of other species studied where males display parental behaviors, OT\IT signaling promoted paternal care similar to *A. ocellaris*. For example, in the bi-parental common marmoset both low (1.0 μg) and high (5.0 μg) doses of OT decreased the amount of food refusals to offspring in fathering males, implying that increased OT levels make fathers more attentive to their offspring (Saito and Nakamura, 2011). Similarly, in the bi-parental teleost fish, *Amatitlania nigrofasciata*, IT neurons in fathering males displayed increased c-Fos expression, suggesting higher activity of IT positive cells during parental care. Further, blockade of IT signaling in this species reduced total parental effort (O'Connell et al., 2012). Additionally, in the bi-parental California mouse *Peromyscus californicus*, plasma OT levels varied across the reproductive cycle and were higher in expectant fathers than non-expectant fathers. Specifically, plasma OT rose 1 day post copulation and remained elevated for 15 days (Gubernick et al., 1995). Together, these data suggest that the role of IT/OT signaling in *A. ocellaris* is similar to other vertebrate species independent of the parental strategy employed.

4.2. IT and aggression

One possible interpretation of the IT antagonist result on paternal behavior is that the dose that was used non-specifically impaired brain function (e.g., made the animal sick, or generally reduced activity) rather than specifically interfered with neural circuitry, which supports parenting behaviors. However, this non-specificity interpretation seems unlikely given that in separate trials, using the same dose, the IT antagonist had no influence on aggression, submissive or affiliative behaviors during the dyadic contests. If the result was non-specific, the IT antagonist would have been expected to impair behavioral performance in the dyadic contests as well, however, no effect on behaviors was observed. Consistent with data shown here, in the closely related damselfish *Stegastes leucosticus*, IT injections had no effect on the number of aggressive displays directed towards an intruder, while AVT increased aggression in the same experiment (Santangelo and Bass, 2006). Similarly with findings presented here in *A. ocellaris*, these data suggest a conserved role of IT signaling in paternal care and a limited function in aggression and dominance. However, it is important to note that we did not test aggression in the context of parenting. Had males been tested for aggression while also exhibiting parental care by using the introduction of a stranger male, or nest predator, different results may have been observed.

4.3. AVT and parental care

To the best of our knowledge, this is the first study to find that blockade of the AVT V1a receptor increases parental behavior in a vertebrate species. This result was surprising as parental effort is already extraordinarily high in *A. ocellaris*, and therefore it was unclear whether or not it would be possible to further increase paternal effort. Additionally, in previous studies exploring the role of AVT in parental effort the opposite result was found. In meadow voles, a 3 ng dose of AVP increased paternal behaviors in previously non-parental males, but had no effect in experienced males, while an AVP antagonist reduced care (Parker and Lee, 2001), the opposite effect found in this current study. Similarly, in two species of pipefishes, AVT was higher in parenting males compared to those that were not parenting (Ripley and Foran, 2010). It is possible that AVT signaling has a different role in *A. ocellaris* as compared to other species as a result of their unique life history characteristics. More species encompassing a broad diversity of social systems and parental strategies need to be explored in order to identify the specific function of AVT signaling in the regulation of parental care.

The differences between data presented here, and results from meadow voles and pipefishes could be related to the fact that the form of parental care exhibited by these species differs from those seen in *A. ocellaris*. Meadow voles directly care for live young (Parker and Lee, 2001). The social bond is different than what is observed in *A. ocellaris* where males provide care only during egg development. Hence, it is possible that AVT plays an important functional role in the bond between sire and offspring and consequently facilitates paternal care. Pipefishes also display dissimilar parental dynamics to *A. ocellaris*. Pipefishes are the only vertebrates where males possess a brood pouch. Offspring are transferred from the female to a placenta like structure within the male where eggs are reared (Ripley and Foran, 2010). In contrast to *A. ocellaris*, the paternal investment of pipefishes is not high levels of behavioral acts, but high demand of the physiological needs of the offspring. This suggests that AVT may have differing roles in the regulation of paternal behaviors across vertebrate species depending on the type of parental care exhibited, and/or the intensity of other competing social behaviors.

We speculate that in *A. ocellaris*, blockade of AVT receptors may have increased paternal effort indirectly by decreasing attentional resources directed towards vigilance behaviors such as territory and nest defense. AVT signaling enhances aggression in several teleost species. In the blue-head wrasse (*Thalassoma bifasciatus*) and burtoni cichlid (*Astatotilapia burtoni*), AVT signaling is critical for social dominance, where administering an AVT receptor antagonist inhibited dominance ascension (Huffman et al., 2015; Semsar et al., 2001). Similarly, in *A. ocellaris*, blockade of AVT reduced aggression and led to a higher probability that a fish would display subordinate behavior in a dyadic aggression trial (Yaeger et al., 2014). In a vigilance task trial given to human males, individuals that received 20 IU of AVP via nasal spray showed increased brain activity to a tone stimulus task compared to the placebo (Fehm-Wolfsdorf et al., 1988), supporting the hypothesis that AVT/AVP signaling is an important response for vigilance behaviors. In this present study, no nest predators or intruders were present, and thus the direct role of AVT in behaviors other than parental care was not tested. Future research on this topic is needed to broaden our understanding of the specific role AVT signaling plays in the regulation of vigilance behaviors in *A. ocellaris*.

4.4. Limitations

One limitation of the AVT antagonist data for evaluating effects on paternal behavior (Experiment 1) is that the treatment occurred after animals had received an acute injection of IT antagonist and it is possible that a prolonged effect of the IT antagonist interacted with the AVT antagonist to produce the effect observed here. Recall that at least 28 days separated treatments which we assumed would be sufficient to wash away IT effects, but we did not evaluate this empirically, and to the best of our knowledge there is no information from the literature to evaluate the likelihood of prolonged interactions of an acute IT antagonist with an AVT antagonist on paternal behaviors. The reason the antagonists were administered serially rather than in a counterbalanced fashion is because it was our intention to establish enough information for a single treatment before moving to the next. The treatments were evaluated within subjects, and each subject had their own spawning cycles that varied over the course of several months, hence it is not as if treatments could be applied to a number of individuals at once. Had we not observed a response to the IT with the first injection, we would have changed the dose before moving to the next treatment. Although it is beyond the scope of the present study to administer the AVT antagonist in absence of the IT antagonist, or in a counterbalanced fashion, we wish to alert the reader to this potential confound in our analysis.

In this current study we used peripheral injections of AVT V1a and IT receptor antagonists which are purported to block receptors in the brain, but where those receptors are located, and how they function to influence the cascade of physiological changes associated with social behaviors was not explored. While studies have identified a variety of brain regions involved in parental care, the pre-optic area of the

hypothalamus (POA) has been consistently implicated. Within the POA, AVT and IT neurons project directly to other parts of the brain and to the pituitary, where peptide release is involved in the regulation of gonadal steroid hormones. Therefore, variation and the size and number of AVT and IT containing neurons may influence gonadal steroidogenesis. While peptide release influences production of gonadal steroid hormones, AVT and IT neurons in the brain also contain hormone receptors, and thus circulating levels of gonadal steroids also affect the function of AVT/IT neurons. Paternal behaviors are likely mediated by a reciprocal interaction between the neuropeptides and gonadal hormones and future work is needed to identify the specific molecular cascades that connect AVT/IT signaling to high levels of paternal care exhibited by *A. ocellaris* males.

5. Conclusion

We used nonapeptide receptor antagonists to block signaling while monitoring paternal behaviors during egg rearing in *A. ocellaris*. Blockade of IT signaling reduced male parental effort, but had no effect on aggression, submission or affiliative behaviors in separate dyadic trials suggesting that the reduced parental care was not a trivial, non-specific effect of the IT antagonist dose. Conversely, blockade of AVT signaling increased male parental effort. Taken together with previous studies highlighting the role of AVT in aggression, we speculate that AVT blockade may have increased parental effort indirectly by shifting attentional resources away from vigilance and aggression, and thus allowing more effort to be directed towards parental care, though empirical data would need to be collected before the AVT vigilance hypothesis can be confirmed.

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