



## Neuropeptide regulation of social behavior in a monogamous cichlid fish

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### ABSTRACT

The nonapeptides arginine vasopressin (AVP; including its non-mammalian homolog arginine vasotocin, AVT) and oxytocin (OT; including its non-mammalian homologs mesotocin, MT, and isotocin, IT) regulate social behavior, including aggression and reproduction, via receptors conserved across vertebrates. In monogamous prairie voles, the vasopressin and oxytocin pathways are crucially important for pair-bond formation, specifically by influencing affiliative behavior toward the mate and aggression toward non-mates. Monogamous social systems are found in diverse taxa. We hypothesized that the AVT/IT pathways are associated with mating behavior in monogamous teleost fishes. We used the monogamous convict cichlid, *Amatitlania nigrofasciata*, to test this idea. In the first experiment, we treated males with a general nonapeptide receptor antagonist during pair-bond formation. Control males were treated with vehicle. On the first day of treatment we observed a significant reduction in both affiliative behavior toward the potential mate and aggression toward neighbors. However, the antagonist did not prevent the pair-bond from forming and the behavioral effects disappeared on subsequent treatment days. In the second experiment, we administered on three consecutive days the AVP/OT receptor antagonist to males that were in an established pair-bond. In established pairs, male affiliation towards the mate and aggressive behavior towards territorial neighbors were not affected by the antagonist. Our results indicate that the basic social behaviors typically mediated by the AVP/OT pathways may provide the building blocks necessary for monogamous mating behavior.

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### 1. Introduction

Mating behavior is highly variable among vertebrate species. General patterns of mating behavior include monogamy, in which a female and a male form a social bond, mate exclusively with each other, and typically cooperate to care for their offspring, and various forms of promiscuity, in which individuals of one or both sexes mate with more than one member of the opposite sex [1,2]. Different patterns of mating behavior are broadly distributed throughout vertebrate taxa [2], but surprisingly little is known about the molecular and neural mechanisms that underlie mating behavior, and whether these mechanisms are shared across diverse vertebrate lineages. Much of what we know about the molecular and genetic basis of monogamous mating behavior has come from studies conducted on mammals [3]. Winslow et al. [4] defined monogamous behavior as (1) selective affiliation with the mate, (2) paternal care, and (3) aggressive defense of the mate against conspecifics. The prairie vole, *Microtus ochrogaster*, is a monogamous rodent that differs from a polygamous species, the meadow vole, *Microtus*

*pennsylvanicus*, in the fore- and midbrain distribution of receptors specific for the neuropeptide hormones arginine vasopressin (AVP) and oxytocin (OT) [5,6]. Males selectively affiliate with a mate and behave aggressively toward non-mates after mating with a particular female. AVP has been found to be both necessary and sufficient to produce both selective aggression and partner preference in males [4]. Male prairie voles injected with an antagonist to the V1a vasopressin receptor before mating failed to develop elevated levels of aggression and also failed to exhibit a post-mating preference for the female mate [4]. Continuous intra-cerebroventricular administration of AVP increased aggression in males that had not mated with females and also caused them to prefer ovariectomized females with which they spent time but had not mated [4].

The role of arginine vasotocin (AVT, the AVP homolog in non-mammalian vertebrates) in aggression and pair formation has also been examined in another major tetrapod lineage [7]. In the highly social and monogamous zebrafish, *Taeniopygia guttata*, aggression decreases with time when animals are group-housed, but is higher in paired males than in unpaired males. Kabelik et al. [7] administered a mixture of V1 antagonists directly into the lateral ventricle and observed a decrease in aggression in unpaired males on the first day of group formation, when aggression was performed as competition over mates. However, paired males increased aggression after treatment with the same drug. The drug had no effect on courtship

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behavior or general arousal or activity. Although pair bonding occurred in the absence of V1a-type receptor stimulation, the action of AVT on aggression may nevertheless indirectly impact aspects of pairing under natural conditions.

No previous study has examined the possible role of neuroendocrine mechanisms in mediating pair formation and maintenance in a non-tetrapod lineage. Actinopterygii (ray-finned fishes) represent such a lineage and exhibit a stunning diversity of mating systems, including monogamy [8]. AVT has been found to mediate several aspects of social behavior in fishes [9,10], and could potentially play a role in pair formation in monogamous fish species. Exogenous AVT increased courtship in male bluehead wrasses, *Thalassoma bifasciatum*, but treatment with a V1a receptor antagonist had the opposite effect [11]. AVT was necessary to assume territorial status in both males and females [12]. In territorial male Beaugregory damselfish, *Stegastes leucostictus*, AVT administration resulted in an increase in aggressive behavior toward intruders in a dose-dependent manner, and injection with an antagonist reduced aggressive behavior [13]. In white perch, *Morone americana*, ventricular administration of AVT increased courtship behavior in males [14]. In the peacock blenny, *Salaria pavo*, courting females express elevated levels of AVT mRNA in the brain [15]. In the weakly electric fish *Apteronotus leptorhynchus*, intra-peritoneal AVT injections increased courtship chirps but inhibited agonistic chirps in males [16]. In some fishes, AVT has an inhibitory effect on social behavior. In the plainfin midshipman, *Porichthys notatus*, courtship vocalizations in courting male morphs were inhibited by AVT [17]. AVT administered ventricularly to highly social individual male goldfish, *Carassius auratus*, inhibited social approach toward same-sex conspecifics, but treatment with an AVT receptor antagonist stimulated social approach [18]. The differences in AVT function in different species might be the result of differential expression of AVT mRNA in different regions of the brain. Such a difference has been found to exist between males of different social classes within a single species [10].

OT is a neuropeptide that originated along with AVT/AVP from a gene duplication event in early vertebrate evolution [19]. Its fish and avian/reptilian homologs are isotocin (IT) and mesotocin (MT), respectively. OT has numerous physiological roles, particularly in reproduction in female mammals (e.g., inducing parturition and subsequent milk let-down). Not surprisingly, and in contrast to AVP/AVT, OT has been associated with female behavior. Specifically, in the context of social affiliation in voles, OT has been suggested to modulate mostly female behavior [20,21]. Similarly, in the highly social zebrafish, *Taeniopygia guttata*, the administration of an OT antagonist reduced affiliative behaviors with familiar social partners, and opposite effects were observed after central infusions of MT in a mostly female-specific manner [22]. However, OT function does not appear to be limited to females [23], as differences in effective dosage of pharmacological agents [4,20] and length of observation relative to latency of effects [24] may also explain some of these apparently sex-specific effects. That AVT and IT functions do not have to be exclusive to one or the other sex has been shown elegantly in the plainfin midshipman [17], where AVT and IT can have effects typical of one sex in the same individual: AVT affected male-typical behavior and IT affected female-typical behavior in both females and sneaker males, which do not actively court females and act female-like. In male goldfish, *Carassius auratus*, intracerebroventricular IT increases social approach to conspecific males [18]. Exogenous IT had no effect on aggressive behavior in the Beaugregory damselfish, *Stegastes leucostictus* [13].

Cichlids are one group of fishes that are extremely diverse [25], and the group is well known for its variety of phenotypes in ecology, anatomy, and behavior, including mating behavior [26]. In cichlids from Central America, monogamy is the predominant mating system [27,28]. In order to determine whether the neuropeptides AVT and/or IT play a role in monogamous mating behavior in fishes, we chose a well-studied model cichlid from Central America, the convict cichlid, *Amatitlania nigrofasciata*. The convict cichlid is a small, robust species,

with a history of use in behavior studies, including studies of pair formation [29–32].

In mammals, three receptors are generally known to respond to binding of AVP: V1a, V1b, and V2. V1a is known to modulate social behavior [33], V1b is best known for stimulating adrenocorticotrophic hormone secretion from the pituitary gland in response to stress [34], and V2 for stimulating water reabsorption in the kidneys [35]. However, the effects of each receptor are not limited to one function. For example, V1b is also known to be involved in social interpretation of olfactory cues [36]. OT is known to have one receptor. Recently, several receptors homologous to the mammalian AVP/OT receptors were identified in fishes: V1a1, V1a2, and V2, as well as a receptor for IT [37,38].

In the present study, we test the effects of these pathways on the formation and maintenance of pair-bonds in the convict cichlid by measuring affiliative behavior toward a potential mate and aggression toward non-mates. Because little is known about the specific functions of these receptors in teleost fishes, we used an antagonist known to be widely affective across multiple receptors in the AVP/OT pathways [39].

## 2. Methods

### 2.1. Animals

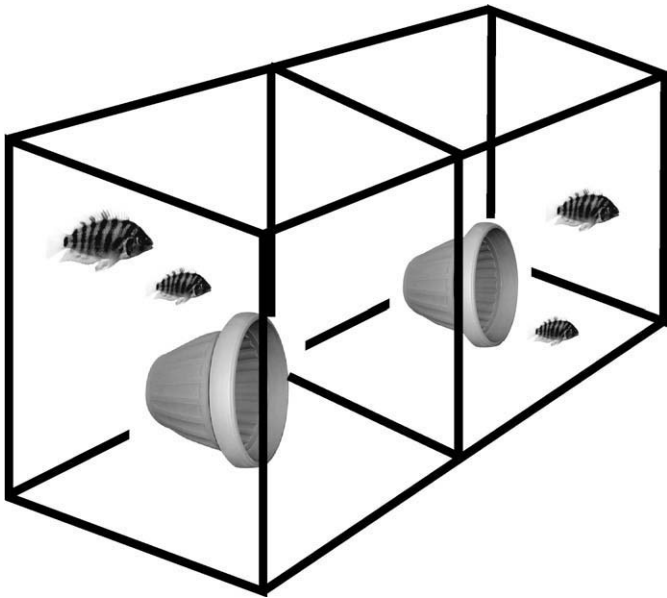
Adult *A. nigrofasciata* were obtained through the pet trade or bred in the laboratory from animals purchased in the pet trade. Fish were held in 230 L stock tanks in single-sex groups on a 12:12 h photoperiod, immediately preceded and followed by a 10-min period of illumination with dim incandescent lights to simulate periods of dawn and dusk. The holding water was maintained at 28 °C and pH = 8.0. Mean body sizes of females was 3.71 (range: 2.11–5.72) g, 44 (range: 37–51) mm SL, and of males was 15.23 (6.96–38.82) g, 70 (55–93) mm SL. We noticed no obvious behavioral differences among animals based on body size (which is also a proxy of age). Most individuals were of the normal “barred” color morph [40], but a small number were leucistic, and pink in color. All pairings were made between individuals of the same color phenotype [41]. Otherwise, each fish was randomly paired with a potential mate from a different source. The male was larger than the female in each pair.

### 2.2. Experimental setup

Experiments were performed in 113 L aquaria that were divided into two compartments by a transparent, perforated partition extending between the front and back panel of the tank (Fig. 1). One male–female pair was held in each compartment with one shelter/spawning site (a clay pot or a short piece of PVC pipe) and a thin layer of sand substrate. In each experiment, the focal pair occupied one compartment and the adjacent compartment contained a stimulus pair that was not involved in an experiment at that time. A pilot study in which small groups were held in a larger, semi-natural environment indicated that the natural process of pair bonding commences when females begin courting a particular male [29–31], and that the pair-bond had formed when the male stopped responding receptively to the courtship of other females and instead began attacking them. The compartmentalized experimental enclosure stimulated pair-bond formation in a way that males typically immediately identified their co-housed female as a mate and behaved aggressively to both the male and the female on the other side of the partition [29], resulting in an instantaneous pair-bond.

### 2.3. Pharmacological manipulations

Pharmacological administrations were similar to those used by Semsar and Godwin [12]. The V1a/V2 and OT receptor antagonist



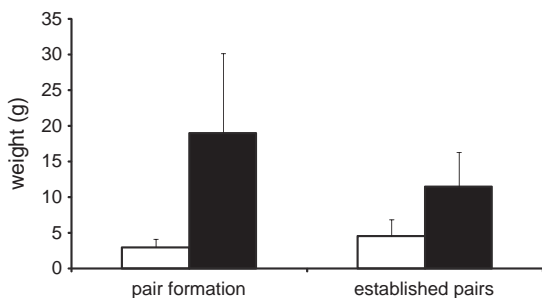
**Fig. 1.** Experimental set-up of test aquarium. A focal pair of convict cichlids was placed on one side of a perforated transparent partition, separated from a stimulus pair that was on the other side of the partition.

([ $\beta$ -Mercapto- $\beta$ ,  $\beta$ -cyclopentamethylenepropionyl<sup>1</sup>, O-Et-Tyr<sup>2</sup>, Val<sup>4</sup>, Arg<sup>8</sup>]-vasopressin, Sigma, St. Louis) was injected intra-peritoneally using insulin syringes at a dose of 3.2  $\mu$ g/g fish body weight, dissolved in a 0.35  $\mu$ g/1  $\mu$ l phosphate buffered saline solution. Control treatments were made by injecting an equivalent amount of phosphate buffered saline. The experimenter was blinded to which fish were getting the treatment or the control.

#### 2.4. Experimental treatments

Two experiments were performed to compare the role of neuropeptides on the pair-bond at two different points in the reproductive cycle. The first experiment tested the role of AVT/IT during pair formation by introducing individuals that had no previous experience with one another. The second experiment tested the role of AVT/IT during pair maintenance by using pairs that had been previously established.

In the pair formation experiment, individuals were held in single-sex groups for a minimum of 14 days and then placed in one half of an aquarium with a member of the opposite sex. Females were chosen based on the appearance that they were physiologically ready to spawn, as indicated by bright orange or red coloration on the abdomen [42], swollen abdomen, and vigorous behavior. Males were chosen indiscriminately. On the first day of experimentation, each male and female was removed from their single-sex tank at 09:00 h, and then weighed (Fig. 2) and measured. Next, males were



**Fig. 2.** Body masses (g) of females (open bars) and males (filled bars) in the pair formation and pair maintenance experiments, as determined immediately before experimentation.

quickly injected intra-peritoneally either with saline ( $n=6$ ) or AVT/IT receptor antagonist ( $n=6$ ). No drugs were administered to females. Both fish were placed into a test aquarium. We conducted 10-min behavioral observations of the focal pair approximately 1 h after injection, a time at which the antagonist would be expected to be effective based on previous research on duration of effectiveness in a similar antagonist [13, Godwin pers. comm.]. On days 2–4 of the experiment, both the male and female were removed from their test compartment at 09:00 h, the male was quickly injected with the same solution used on day 1, both fish were returned to their test tank, and a behavioral observation followed 1 h later.

In the pair maintenance experiment, pairs were formed by housing one male and one female alone together in a compartment for a period of at least 20 days. We noticed no obvious differences among pairs that had been together for 20 days and those that had been together for longer periods of time. Reproductive state was somewhat standardized by attempting to choose pairs that appeared to be preparing to spawn as evidenced by an enlarged genital papilla in the female and an apparent increase in courtship behavior. At 09:00 h on the day before observations began, the male and female of each pair were removed from their compartment, weighed (Fig. 2), measured, and then returned to their original compartment. Beginning the following day, behavior was recorded through one 10 min focal observation performed between 09:15 and 12:00 h each day for three consecutive days without manipulation (pre-treatment). On the fourth day, both the male and female of each experimental pair were removed from their compartment and the male was rapidly injected with either AVT/IT antagonist ( $n=7$ ) or saline ( $n=5$ ). Both fish were then placed back into their original compartment and observed approximately 1 h later. Injections and observations were performed again on each of the following two days for a total of three days of treatments.

All possible steps to minimize stress in the subjects were taken, including limiting handling time to a few seconds, and carefully observing all social relationships and separating individuals if excessive aggression posed a threat to the health of another fish. Methods were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin, and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Office of Science and Health Reports, Bethesda, MD).

#### 2.5. Behavioral observations

In both experiments, each pair was observed in a series of daily 10 min observation periods conducted between 09:15 and 12:00 h. We developed a novel ethogram, building upon classical ethological descriptions of cichlid mating behavior [43,44,see Results], and used focal sampling and continuous recording [45] to score each bout of behavior performed by each pair-member, and which fish the behavior was directed towards. A bout was defined as a series of one or more consecutive identical acts. A bout ended when the behavior stopped for >1 s or as soon as a different behavior was performed. In addition, time budgets were interpreted by scan sampling and recording the instantaneous behavior of each individual at 1-minute intervals [45]. Time budgets included some behavioral states that were not recorded during continuous recording of bouts (e.g., swimming, resting on the substrate, etc.).

#### 2.6. Statistical analyses

In the pair formation experiment data sets frequently failed Levene's test for equal variances and Kolmogorov–Smirnov tests for normality ( $p \leq 0.05$ ), which are required for parametric analysis. These deficiencies were not remedied through log or square root data transformations so male aggressive behavior performed toward



neighbors and affiliative behavior performed toward the potential mate were compared between treatments on each day with non-parametric Mann–Whitney tests. Observations suggested that once spawning had commenced affiliative behavior may have been partially replaced with reproductive behavior, so tests of affiliation were performed a second time while including reproductive behavior. Because males are known to prefer large females, females are known to prefer large males, and individuals pair assortatively by body size in laboratory groups and in nature [46–48], the role of relative body size in pair formation was assessed by testing for association between female/male mass ratio and aggression and for association between female/male mass ratio and affiliation with Spearman rank correlation tests on each day of the experiment. Additionally, in an attempt to further investigate the effect of behavior performed by the female mate, non-parametric Wilcoxon signed ranks tests were used to compare males' with females' performance of aggression and affiliation. In order to determine if any observed effects on aggressive or affiliative behavior were due to general changes in overall activity (possibly as a result of peripheral effects caused by the antagonist blocking the V2 receptor), proportions of time spent swimming, foraging, displaying, and performing aggressive or reproductive behavior were combined. The resulting data sets passed all tests of normality and equal variance, so they were compared between treatments using a repeated measures general linear model in SPSS, with day as a within-subjects variable, treatment as a between-subjects factor, and female/male mass ratio and overall female activity as covariates. In the pair formation experiment the presence of offspring was a result of experimentation and the numbers of pairs that spawned during the four days of the experiment were compared between treatments using Fisher's exact test.

In the *pair maintenance* experiment, three dependent variables were considered: aggression toward neighbors, affiliation toward mate, and overall activity (as defined above). As in the pair formation experiment, analyses were run a second time with reproductive behavior included in measures of affiliation. All dependent variable data sets passed Kolmogorov–Smirnov tests for normality ( $p \geq 0.292$ ), and all but one passed Levene's test for equal variances (affiliative and reproductive behavior on day 4:  $F(3,8) = 7.175$ ,  $p = 0.012$ ; all other dependent variables:  $p \geq 0.094$ ). Therefore, data were compared between treatments using a doubly multivariate repeated measures general linear model in SPSS, with day as a within-subjects variable, treatment as a between-subjects factor, and female/male mass ratio and overall female activity over days 4–6 as covariates. In contrast to the pair formation experiment, in established pairs the presence of offspring was included as a fixed factor in the general linear model. Three of five pairs in the saline control group (all on day 2, during the pre-treatment period), and four of seven pairs (one on day 1, and three on day 2) in the receptor antagonist treatment group produced offspring during the experiment.

Alpha was set at 0.05 and all tests were two-tailed. Multivariate statistics are reported as results of Hotelling's trace tests.

### 3. Results

#### 3.1. Developing a novel ethogram

We established a new ethogram for the convict cichlid, based on behavior observed in a pilot study in which small groups were held in a large, semi-natural environment (Table 1). In a pilot work to distinguish between aggressive behavior and affiliative behavior it became obvious that a 'bite' was not typically a form of aggression when performed toward the mate during the advanced stages of courtship and brood care. The action was performed at a lower rate of speed and with less intensity than when it was performed toward a non-mate. We could not identify any other distinguishing differences in this basic motor pattern, which suggests that the communication

**Table 1**  
Ethogram of behavior observed in convict cichlids relevant to monogamous pair formation.

<i>Aggressive behavior</i>	
Bite	Subject contacts another fish with mouth open, sometimes closing it, sometimes causing physical damage.
Charge	Rapidly approaches another fish, stopping suddenly before making physical contact. Often with mouth open. Locomotion occurs in a straight line only.
Chase	Rapidly follows another fish, sometimes making contact in the form of a bite (if not separated by tank partition). Trajectory may change depending on movement of target fish.
Synchronized attack	Male and female attack neighbors at the same time, side by side.
<i>Affiliative behavior</i>	
Lateral display	Median fins erect. Subject stationary or slowly circling the other fish. Vertical axis of body often oblique. Often performed in conjunction with operculum flare.
Frontal display	Subject is facing another fish. Mouth and opercula are open and branchiostegal membrane is extended. Body may take a sigmoid shape in sagittal plane.
Tailbeating	Subject remains stationary and performs exaggerated sinusoidal swimming motions while positioned next to another fish.
Circling	Subject swims in circles. Usually before spawning, and usually around the spawning site.
Greeting	Male and female approach each other simultaneously and swim past each other, often erecting opercula and brushing against one another (Baylis 1974).
Approach	Subject swims in the direction of another fish.
Affiliative bite	Subject softly touches/closes mouth on another fish. Mouth can be open or closed. May or may not bite down on target's body. Distinguished from 'bite' when the receiver does not flinch, flee, or respond aggressively.
<i>Reproductive behavior</i>	
Dig	Moving sand with mouth or fins, resulting in a pit.
Quiver	Subject shivers/twitches, starting at head, passing lateral waves down the body.
Skim	Subject slowly passes over spawning site with genital papilla in contact with spawning site.
Nip off	Subject bites the spawning site, apparently cleaning it in preparation for egg deposition. (Nip eggs: similar, but occurs after eggs have been laid. Also recorded when parent uses mouth to pick up fry after they have hatched.)
Fan	Subject hovers above eggs, pushing water over them using the pectoral fins.

function of some signals is dependent on social context in this species. An aggressive 'bite' and an affiliative 'bite' were operationally distinguished easily by observing the response of the receiving fish. If the receiving fish flinched, fled, or responded aggressively then we considered the behavior to be aggressive; if it did not, we considered the behavior to be affiliative. In the same way, an 'approach' was very similar in modal action pattern to a 'charge', but usually did not result in flight, defense, or appeasement.

Aggressive behavior typically consists of both display and attack modal action patterns, but under the experimental conditions aggressive displays were typically not performed due to the fact that the aquarium partition accelerated pair formation [29], so that behavior toward a 'mate' was almost always affiliative, and behavior toward neighbors consisted of high-intensity attacks. Aggression toward non-mates frequently took the form of swimming rapidly against the partition and biting it, as if trying to reach the fish on the other side. Bouts of this form of aggression seemed much longer in duration than under semi-natural conditions that permitted contact between individuals.

Affiliative behavior included several modal action patterns (Table 1). As stated above for affiliative 'bite', affiliative behavior generally could be characterized by the response of the receiver, which was either absent or was another affiliative action. Bouts of affiliative behavior were typically relatively brief. If the pair spawned

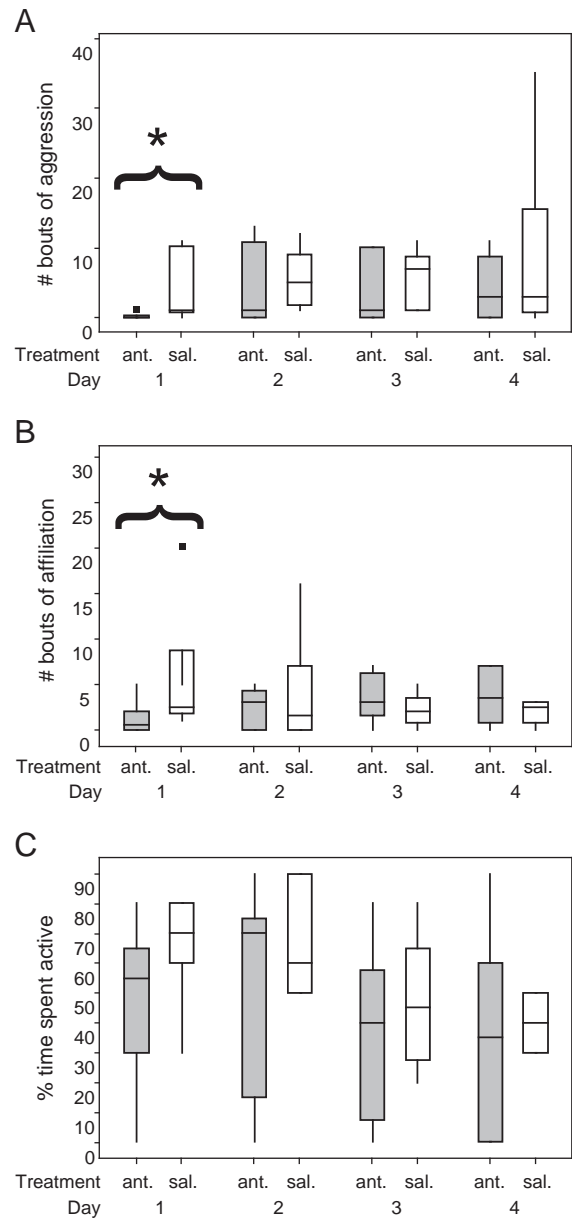
during the experiment, then affiliative behavior appeared to be partially replaced with reproductive behavior, which involved directly producing or caring for offspring. Despite the fact that these two categories of behavior included different modal action patterns, in the current experiment they were interpreted as having the same function (reproduction), only occurring at different stages in the mating and reproduction process. Behavioral coordination typified pairs in the advanced stages of reproduction. They performed mutual displays, 'greeting ceremonies' in which the two fish swiftly swam by one another and exchanged frontal displays, and 'dual attacks', in which a bonded male and female attacked their neighbors together in synchrony.

### 3.2. Pair formation

During pair formation, inhibiting the AVT/IT pathways in males resulted in lower territory defense and mate affiliation on the first day of pair formation than in controls (Mann–Whitney test: aggression:  $Z = -2.268$ ,  $p = 0.023$  on day 1;  $p \geq 0.170$  on each of days 2–4; Fig. 3A; affiliation:  $Z = -2.038$ ,  $p = 0.042$  on day 1,  $p \geq 0.617$  on each of days 2–4; affiliation and reproduction:  $Z = -2.038$ ,  $p = 0.042$  on day 1,  $p \geq 0.369$  on each of days 2–4; Fig. 3B). Behavior of the female mates also may have affected male defense behavior, because the significant difference in male aggression on day 1 disappeared when male–female synchronized attacks were included in the analysis ( $Z = -1.608$ ,  $p = 0.108$ ). Non-parametric comparisons of male and female behavior found no significant differences between the sexes in aggression (including synchronized attacks) performed on day 1 in the saline treatment ( $Z = -0.271$ ,  $p = 0.786$ ), but males performed significantly fewer aggressive bouts on day 1 in the antagonist treatment ( $Z = -2.032$ ,  $p = 0.042$ ). On any given day, female/male mass ratio was not correlated with either aggression (Spearman rank correlation:  $-0.161 > r_s > 0.091$ ,  $p \geq 0.618$ ) or affiliation/reproduction ( $-0.210 > r_s > 0.293$ ,  $p \geq 0.355$ ), indicating that during pair formation the difference in body size between a particular male and female did not affect the male's pair-bonding behavior toward that female. The antagonist's reduction in aggression and affiliation were not a result of a general reduction in activity (Fig. 3C); no factors had a significant effect in the general linear model (day:  $F(3,24) = 0.618$ ,  $p = 0.610$ ; treatment:  $F(1,8) = 3.124$ ,  $p = 0.115$ ; ; female/male mass ratio:  $F(1,8) = 0.977$ ,  $p = 0.352$ ; female activity:  $F(1,8) = 2.919$ ,  $p = 0.126$ ; day\*treatment:  $F(3,24) = 0.777$ ,  $p = 0.518$ ). There was no significant difference between treatments in the number of pairs that spawned within the four days of experimentation (Fisher's exact test:  $p = 1.0$ ). Two of six saline-treated pairs spawned on days 3 and 4 and one of six antagonist-treated pairs spawned on day 3, so there was no difference in latency between the control and treatment groups.

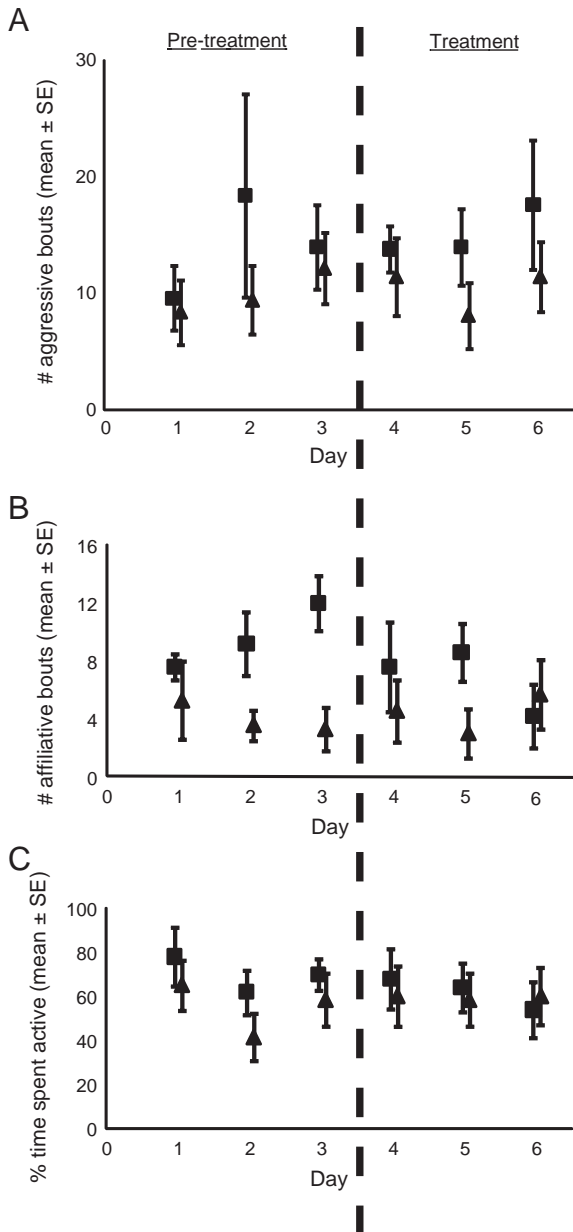
### 3.3. Pair maintenance

In order to determine if the effect of the AVT/IT antagonist differed on sequential days of administration, the general linear model was first performed by including only the three days of injections, and not the initial three days of pre-injection observations (Fig. 4). Inhibiting the AVT/IT pathway in males did not significantly affect any aspect of behavior ("treatment" in Table 2). We then tested whether female activity and/or the presence of offspring might have an effect on male behavior (independent of a treatment effect). When the model excluded reproductive behavior from measures of social affiliation in males, we found that indeed only female activity and presence of offspring did have significant effects in the GLM (Table 2). No significant within-subjects univariate effects were found. Finally, only female activity and the presence of offspring had strong between-subjects effects on affiliative behavior, but not on other male behavior. When measures of social affiliation included both affiliative behavior and reproductive behavior, we obtained similar results (Fig. 4B).



**Fig. 3.** Behavior of males during pair formation. Box plots representing medians and first and third quartiles (Q1 & Q3) of numbers of (A) aggressive bouts performed toward members of a neighboring pair, (B) combined affiliative and reproductive behavior performed toward a potential mate, and (C) overall activity in males injected with either a nonapeptide antagonist (ant.) or saline (sal.). Whiskers represent upper and lower limits [(Q3 – Q1) × 1.5]. Squares = outliers. Asterisks =  $p < 0.05$ .

In order to determine whether behavior performed on days before treatment commenced differed from behavior after the onset of treatment we built a second general linear model (Table 3). Data across the three days on which the fish were injected were pooled and compared to data pooled from the first three days on which no injections were performed. When the GLM excluded reproductive behavior from measures of social affiliation in males, multivariate tests produced results similar to those of the first analysis, revealing that again only female activity and presence of offspring showed significant effects in the GLM. No effects of day were found in within-subjects univariate tests. Finally, female activity, female/male mass ratio, and the presence of offspring exerted significant between-subjects effects on affiliative behavior, with the latter also affecting male aggression. We then conducted the analysis a second time, but now included both affiliative behavior and reproductive behavior in



**Fig. 4.** Behavior of males in established pairs. (A) Mean number of aggressive bouts performed toward members of a neighboring pair, (B) combined affiliative and reproductive behavior performed toward an established mate, and (C) overall activity in males injected with either a nonapeptide antagonist (triangles) or saline (squares). Error bars represent standard error.

measures of social affiliation (Table 3). We did find an effect of day, although only on the combined measure of affiliative behavior and reproductive behavior, suggesting that injection of both drug and saline affected reproductive behavior.

**4. Discussion**

We have found that endogenous nonapeptides are involved in producing two primary behavioral components that characterize monogamous pair formation in a fish. Treatment with an AVP/OT receptor antagonist decreased both affiliative behavior performed toward a potential mate, and aggressive behavior performed toward potential territory intruders on the first day of administration, but not on subsequent days. While we cannot conclusively state whether the AVT

**Table 2**

Results of a general linear model that examines male behavior performed after treatments had begun (days 4–6) in the pair maintenance experiment. The results shown are from tests that considered only affiliative behavior, and not reproductive behavior in measures of social affiliation. Significant results are highlighted in bold.

	<i>d.f.</i>	<i>F</i>	<i>p</i>
<i>Multivariate tests</i>			
Day	6,1	1.189	0.606
Treatment	3,4	0.285	0.835
Female/male mass ratio	3,4	1.652	0.312
Female activity	3,4	15.549	<b>0.011</b>
Presence of offspring	3,4	21.439	<b>0.006</b>
<i>Univariate tests</i>			
Day on aggression	2,12	0.089	0.915
Day on affiliation	2,12	1.058	0.377
Day on overall activity	2,12	1.898	0.192
<i>Between-subjects effects</i>			
Treatment on aggression	1,6	0.856	0.391
Treatment on affiliation	1,6	0.211	0.662
Treatment on activity	1,6	0.063	0.810
Female/male mass ratio on aggression	1,6	0.005	0.948
Female/male mass ratio on affiliation	1,6	3.730	0.102
Female/male mass ratio on activity	1,6	0.177	0.688
Female activity on aggression	1,6	0.890	0.382
Female activity on affiliation	1,6	39.560	<b>0.001</b>
Female activity on activity	1,6	0.134	0.727
Presence of offspring on aggression	1,6	3.528	0.109
Presence of offspring on affiliation	1,6	45.502	<b>0.001</b>
Presence of offspring on activity	1,6	0.516	0.499

**Table 3**

Results of a general linear model that compares male behavior performed on days before treatment commenced (days 1–3) with behavior performed after the onset of treatment (days 4–6) in the pair maintenance experiment. Significant results are highlighted in bold.

Affiliative behavior only	<i>d.f.</i>	<i>F</i>	<i>p</i>
<i>Multivariate tests</i>			
Day	3,4	0.986	0.484
Treatment	3,4	0.433	0.741
Female/male mass ratio	3,4	4.190	0.100
Female activity	3,4	10.879	<b>0.022</b>
Presence of offspring	3,4	17.397	<b>0.009</b>
<i>Univariate tests</i>			
Day on aggression	1,6	0.050	0.830
Day on affiliation	1,6	2.921	0.138
Day on overall activity	1,6	1.197	0.316
<i>Between-subjects effects</i>			
Treatment on aggression	1,6	0.920	0.375
Treatment on affiliation	1,6	1.366	0.287
Treatment on activity	1,6	0.481	0.514
Female activity on aggression	1,6	1.523	0.263
Female activity on affiliation	1,6	18.710	<b>0.005</b>
Female activity on activity	1,6	0.468	0.519
Female/male mass ratio on aggression	1,6	0.012	0.917
Female/male mass ratio on affiliation	1,6	7.233	<b>0.036</b>
Female/male mass ratio on activity	1,6	0.125	0.736
Presence of offspring on aggression	1,6	7.264	<b>0.036</b>
Presence of offspring on affiliation	1,6	22.392	<b>0.003</b>
Presence of offspring on activity	1,6	2.768	0.147
<i>Affiliative behavior and reproductive behavior</i>			
<i>Multivariate tests</i>			
Day	3,4	10.864	<b>0.022</b>
<i>Univariate tests</i>			
Day on aggression	1,6	0.050	0.830
Day on activity	1,6	1.197	0.316
Day on affiliation/reproduction	1,6	44.410	<b>0.001</b>

or IT pathway, or both, are involved in this process, the current results demonstrate for the first time the importance of peptide pathways in producing specific types of behavior in a pair-bonding fish.

In a pilot study involving individuals held in large groups under semi-natural conditions, it was determined that a pair-bond had formed when, after repeated courtship by multiple females [29–31], a male stopped responding receptively and started responding aggressively to all but one particular female. The design of the pair formation experiment stimulated and standardized pair formation by presenting a female and a male with a breeding site and potential intruders [29] and allowed a controlled assessment of the effect of the AVP/OT antagonist. Under these conditions, treatment with antagonist had no effect on number of individuals spawning, or on latency to spawn. However, a reduction in affiliative behavior toward a potential mate and in aggressive behavior toward territorial intruders would likely reduce the chances of successful pair formation under natural conditions.

The current study did not detect an effect of the AVP/OT antagonist on male behavior in established pairs. Not surprisingly, the presence of offspring, female activity, and female/male mass ratio all significantly affected male behavior, which suggests that our model performed well overall. However, it is possible that the AVT/IT pathways play a role in pair maintenance as well as in pair formation, but that our experimental paradigm was unable to detect it. For example, familiar neighbors (as used in the current study) may elicit lower levels of aggression than unfamiliar intruders. Conversely, the presence of the partition might elicit prolonged aggression toward neighbors. An effect of the antagonist could have been masked by these factors. There was a high level of variation in the data in the pair maintenance experiment. Notably, there appeared to be higher levels of affiliative behavior in the saline treatment than in the antagonist treatment, both before and after the initiation of injections. Because these differences appeared only sporadically on days 2, 3, and 5, they could not have been the result of the experimental treatment. However, when we combined both affiliative behavior and reproductive behavior in one measure of social affiliation, pre- and post-treatment behavior differed significantly even in the control group, indicating that the injection procedure, but not the antagonist, caused a reduction in reproductive behavior only.

The behavioral paradigm that we constructed for the purpose of evaluating the effect of nonapeptides on mating behavior required that we distinguish between affiliative behavior and aggressive behavior, and resulted in the identification of a new behavior pattern. Previous investigators based their analyses primarily on motor patterns [43,49]. However, a single motor pattern has often been reported to occur in different social contexts [49]. For example, in the past a 'bite', a motor pattern in which the upper and lower jaws are brought together on another individual, has been analyzed exclusively as an act of aggression, irrespective of the stage of courtship [49]. We determined that an affiliative 'bite' is distinct from an aggressive 'bite', despite the similarity in motor pattern. This is consistent with reports of subdued biting being performed in pair-bonded cichlids. Baylis [49] defined a 'bite' in *Amphilophus citrinellus* and *A. zalius* as occurring with or without a violent closing of the jaws around the receiver's anatomy. In these species [49], and in *Herotilapia multispinosa* [44], he noted that just before spawning the 'bites' took the form of an open-mouthed push rather than a true bite typical of aggressive interactions. Interestingly, performance of a 'bite' by a male increased the chance that the female would then perform a quiver, a behavior that precedes spawning. In paired convict cichlids, Williams [29] described males touching their snouts to the female's body, and Meral [30] reported both sexes to perform gentle nips that seemed more like grooming than aggressive nips, which were rare. McKaye [50] reported that "attacks" were performed by *Hypsophrys nicaraguensis* when initiating cooperative broodcare with *Parachromis dovii*, but recently published video footage of this behavior reveals subdued intensity on the part of the

performer, and absence of defensive response on the part of the receiver [51]. It seems likely that previous researchers may not have distinguished between affiliative and aggressive bites because such a distinction was not required to address their particular hypotheses. However, such a distinction was necessary to address the current hypothesis, which required independent comparisons of affiliation and aggression.

The results of the current study are similar to those obtained for monogamous prairie voles [4]. Male prairie voles ventricularly infused with a V1a antagonist failed to develop a typical increase in aggressive behavior after co-habitation with a female, although there was no change in the number of males mounting females or in the number of mounts. Aggression was not reduced in treated males that had previously-established, existing pair-bonds with females. In the current study, AVP/OT antagonist reduced aggressive behavior and affiliative behavior during pair formation, but not in established pairs. That the effect was only significant on the first day of administration is further support for the neuropeptide pathway functioning in pair formation but not in pair maintenance. Infusion with V1a antagonist also blocked partner preference in male prairie voles. When given a choice between the mate and a novel female, treated males equally divided their time between a novel female and their mate instead of spending more time with the mate. Whether a nonapeptide antagonist would block partner preference in convict cichlids has yet to be determined. The results of the current study are also consistent to the results obtained by Kabelik et al. [7] in zebra finches. Males treated with a mixture of V1 antagonists performed decreased levels of aggression on day one of co-habitation, during which males aggressively compete over mates. On subsequent days, the antagonists caused an increase in aggression in paired males defending nests. Therefore, in both convict cichlids and zebra finches, AVP/OT antagonist reduced aggression during pair formation but did not reduce aggression in established pairs. However, although AVP/OT antagonist decreased affiliative behavior in convict cichlids, it had no effect on courtship behavior in zebra finches.

Understanding the evolution of neuropeptide pathways has been complicated by the lack of consistent effects across different species [9]. However, both AVT and IT are known to affect basic social behaviors, such as approach and avoidance, in some species [18]. The elaboration of the function of AVT and IT from basic social behaviors to more complex and species-specific behaviors in particular social contexts may occur through differential expression of either the neuropeptide and/or its receptors in different regions of the brain. Greenwood et al. [10] found that dominant, territorial male cichlids, *Astatotilapia burtoni*, have higher AVT expression in the gigantocellular nucleus of the posterior preoptic area and subordinate males have higher AVT expression in the parvocellular nucleus of the anterior preoptic area. Similarly, Kabelik et al. [7] concluded that the differences in the effect of AVP antagonist in different social contexts were probably due to differences in relative activation of different AVT neuronal populations. Differential expression of AVT or its receptors across brain regions in different social contexts might explain the apparently inconsistent effects of exogenous AVT across different species. Thus, the role of the nonapeptide pathways in regulating social behavior may truly be conserved across taxa.

The general functions of AVT/IT in mediating affiliative behavior and territorial behavior may also be recruited to produce monogamous mating behavior. Under ecological conditions that select monogamous mating behavior, an existing mechanism that produces general social attraction might be co-opted to function in monogamous mate affiliation and partner preference, and existing pathways that function to produce aggressive behavior might be co-opted to produce aggression against brood-site intruders. Such changes in mating system have been proposed to occur as a result of a change in the gene for the V1a receptor, but it is likely that other mechanisms or an interaction of pathways are involved in the formation of the pair-bond [52,53].



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