

# Exogenous vasotocin alters aggression during agonistic exchanges in male Amargosa River pupfish (*Cyprinodon nevadensis amargosae*)

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## Abstract

Pupfishes in the Death Valley region have rapidly differentiated in social behaviors since their isolation in a series of desert streams, springs, and marshes less than 20,000 years ago. These habitats can show dramatic fluctuations in ecological conditions, and pupfish must cope with the changes by plastic physiological and behavioral responses. Recently, we showed differences among some Death Valley populations in brain expression of arginine vasotocin (AVT). As AVT regulates both hydromineral balance and social behaviors in other taxa, these population differences may indicate adaptive changes in osmoregulatory and/or behavioral processes. To test whether AVT is relevant for behavioral shifts in these fish, here we examined how manipulations to the AVT system affect agonistic and reproductive behaviors in Amargosa River pupfish (*Cyprinodon nevadensis amargosae*). We administered exogenous AVT (0.1, 1, and 10  $\mu\text{g/g}$  body weight) and an AVP  $V_1$  receptor antagonist (Manning compound, 2.5  $\mu\text{g/g}$  body weight) intraperitoneally to males in mixed-sex groups in the laboratory. We found that AVT reduced the initiation of aggressive social interactions with other pupfish but had no effect on courtship. The effects of AVT were confirmed in males in the wild where AVT (1  $\mu\text{g/g}$  body weight) reduced the aggressive initiation of social interactions and decreased aggressive responses to the behavior of other males. Combined, these results show that AVT can modulate agonistic behaviors in male pupfish and support the idea that variation in AVT activity may underlie differences in aggression among Death Valley populations.

*Keywords:* Arginine vasotocin; AVT; Neuropeptide; Pupfish; Social behavior; Aggression; Vasopressin; AVP; Hormone evolution; Fish

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## Introduction

The Death Valley region of California and Nevada is home to a monophyletic group of pupfishes that have been diverging in allopatry over the last 400–20,000 years (Miller, 1950). During the Pleistocene, Death Valley contained a large lake and river system that evaporated as the climate became increasingly arid. The pupfish that occupied these waters became isolated in a series of remnant aquatic habitats including freshwater springs, hypersaline marshes, and desert streams (Soltz and Naiman, 1978). The ecological

diversity of these habitats has generated considerable variation in social behaviors in Death Valley pupfishes on both the population and individual levels (for reviews, see Kodric-Brown, 1981; Soltz and Hirshfield, 1981).

The social organization of pupfish populations in Death Valley ranges from territorial breeding systems to spawning in schools. For instance, male Ash Meadows pupfish (*Cyprinodon nevadensis mionectes*) are highly aggressive toward conspecifics as they defend reproductive territories over the substrate (Soltz, 1974). Devil's Hole pupfish (*Cyprinodon diabolis*), on the other hand, show almost no overt aggression between males (reviewed by Kodric-Brown, 1981). The social organization in other populations, however, can be variable as individuals alter their behavior to cope with rapid changes in the conditions of their desert habitat. Amargosa River pupfish (*Cyprinodon nevadensis*

*amargosae*) largely breed in loose aggregations where males show little aggression and regularly court females. Some males in this population, however, establish and defend reproductive territories in the warm, shallow edges of the river. These territorial males are aggressive and infrequently court females (Soltz, 1974). Yet the frequency of these behaviors can vary dramatically during the year as water flow, temperature, and conspecific density change due to flash flooding and desiccation during the extreme Death Valley summer.

Given that behavioral variation in Death Valley pupfishes is primarily expressed as changes in the frequency of agonistic and reproductive behaviors (Liu, 1969), this behavioral variation may be mediated in part by plastic neuroendocrine responses to dissimilar environments. Supporting this idea, we recently found differences in the size of arginine vasotocin (AVT)-immunoreactive parvocellular and magnocellular neurons in the preoptic area in pupfish from two populations in Death Valley (Lema and Nevitt, 2004). AVT and its mammalian homologue, arginine vasopressin (AVP), have been shown to alter social behaviors in a variety of taxa (for a review, see Goodson and Bass, 2001). Exogenous application of AVT or AVP affects diverse behaviors including calling by anuran amphibians (Semsar et al., 1998), song production in birds (Goodson, 1998a; Harding and Rowe, 2003), and parental care in mammals (Parker and Lee, 2001). Exogenous AVT has been shown to modulate courtship and aggressive behaviors in fish as well. For example, in the weakly electric fish *Apteronotus leptorhynchus*, intraperitoneal injection of AVT increased the production of type-I electric organ chirps, a communication signal emitted during courtship and mating, while decreasing type-II chirps that are typically produced during agonistic exchanges (Bastian et al., 2001). In bluehead wrasse (*Thalassoma bifasciatum*), exogenous AVT decreased aggression in territorial males but increased aggression in nonterritorial males (Semsar et al., 2001). Both territorial and nonterritorial males also increased courtship following administration of AVT.

Based on these studies, we hypothesized that AVT might mediate behavioral shifts in agonistic and reproductive behaviors in pupfish. Specifically, we predicted that increased levels of AVT would inhibit aggression in territorial male pupfish while increasing sexual behaviors such as courtship and spawning. Here we tested this idea by exploring how manipulations of the AVT system affected behavior in male Amargosa River pupfish (*C. n. amargosae*). We administered three concentrations of exogenous AVT and an antagonist to the  $V_1$  receptor for AVP (Manning compound) intraperitoneally to males maintained in mixed-sex social groups in the laboratory. We also examined the behavioral effects of AVT on wild male pupfish in the Amargosa River. Some results from these experiments have appeared in preliminary form (Lema and Nevitt, 2002).

## Materials and methods

### *Study site and animals*

Amargosa River pupfish (*C. n. amargosae*) were studied in the Amargosa River near Dumont Dunes, San Bernardino County, CA. The Amargosa River extends for approximately 320 km before emptying onto salt flats on the floor of Death Valley. Over most of its extent, however, the Amargosa River is dry except during floods, and pupfish can only be found in two small sections with permanent water. We studied pupfish from the larger of these sections—a 10- to 12-km stretch where the Amargosa River flows over bedrock before vanishing into permeable desert sands (for descriptions, see Lema and Nevitt, 2004; Soltz and Naiman, 1978). All procedures were approved by the Animal Care and Use Committee of the University of California, Davis.

### *Pupfish behaviors*

We observed a suite of behaviors performed during interactions with other pupfish (Table 1). These behaviors were categorized according to descriptions of motor patterns of *C. nevadensis* and other pupfish species (Barlow, 1961; Liu, 1969; Soltz, 1974). As we were specifically interested in how AVT affects social behaviors, we focused our observations on behaviors related to agonistic and reproductive exchanges. For each social exchange, we documented which individual initiated the social interaction, the sequence of motor patterns involved in the interaction, and the outcome of the interaction. Aggressive behaviors where the focal male initiated a social interaction were thus analyzed separately from the same motor patterns performed when the focal male was responding to an aggressive interaction initiated by a nonfocal fish. Unless specifically stated otherwise, our analysis of agonistic behaviors (i.e., charges, nips, displays) represents behaviors performed when focal males initiated a social interaction.

### *Experiment 1: effects of AVT and a $V_1$ antagonist*

Pupfish were collected on October 18, 2001, by minnow trap and dip net from the Amargosa River and transported to the Center for Aquatic Biology and Aquaculture at the University of California, Davis. Fish were kept in 1.2-m diameter, flow-through tanks that were maintained at 26–28°C under ambient photoperiod until experimental testing.

### *Testing environment*

Two weeks before testing, pupfish were assigned to social groups of three males and four females and transferred to testing tanks (approximately 114 l; 90 cm long × 45 cm wide × 30 cm high). All fish used in the experiments were sexually mature. The bottom of each tank was covered

Table 1  
Description of male pupfish behaviors recorded in this study

Behavior	Description
Aggressive	
Charge	Individual rapidly darts toward another fish with mouth open and median fins folded
Nip	Individual rapidly darts toward another fish with mouth open and contacts the individual
Display	Males approach each other and momentarily pause face-to-face or side-to-side less than one body length apart with the median fins spread
Courtship	
Sidle	Male swims forward and closely alongside a female while contacting her pectoral fin region with his snout
S-shape	Spawning—male and female lie side-by-side on the substrate with their bodies curved, forming an S-shape pattern; the dorsal fin of the male is spread, and the anal fin is wrapped around the female's anal-genital region; usually followed by oviposition
Additional behaviors	
Feeding bites	Individual quickly tilts and takes a mouthful of sand and algae from the substrate; the sand is either immediately spit out, or the fish swims forward a short distance and then expels the substrate from the mouth
Resting	Individual stops swimming and movement of fins and lies motionless on the substratum

Descriptions adapted from Barlow (1961) and Soltz (1974).

with 3 cm of sand, and rocks were placed within each tank for structure. Pupfish males use such structures (e.g., rocks, plant debris) to delineate boundaries between reproductive territories in natural habitats (e.g., Barlow, 1961) and in aquaria (e.g., Itzkowitz, 1978). Testing tanks were maintained at  $28 \pm 0.8^\circ\text{C}$  and 0.4 ppt salinity on a 14:10-h light–dark photoperiod. These physical parameters were within the natural range of salinity and temperature in the Amargosa River (see Lema and Nevitt, 2004).

#### *Hormone administration and behavioral observations*

Because of the small size of pupfish (generally <40 mm standard length), AVT was administered peripherally as a single intraperitoneal injection rather than intracranially. This method has previously been shown to affect behavior in other species of fish (Bastian et al., 2001; Salek et al., 2002; Semsar et al., 2001), amphibians (Semsar et al., 1998), and rodents (Cushing et al., 2001). While peripheral injections of AVT can cause a suite of physiological responses in fish including elevated blood pressure (Conklin et al., 1997), intracerebroventricular injections can have the same effects (Le Mevel et al., 1991, 1993).

Between June 24 and August 25, 2002, we administered hormone solutions to one male from each testing tank. Experimental males ( $n = 10$ ; standard length,  $37.87 \pm 1.59$  mm, mean  $\pm$  SE; body weight,  $1.98 \pm 0.26$  g) were always either the largest or intermediate-sized male in the tank since only these males established and defended territories over the substrate. The smallest of the three males was never used as an experimental subject. All experiments were conducted between 11:00 and 17:00.

To quantify how the administration of hormones changed behavior, we observed pupfish both before and following hormone administration. Behaviors were recorded using The Observer (Version 3.0; Noldus Information Technology) computer software. We first observed the focal male

for 60 min to establish a behavioral baseline. Immediately following this preinjection observation, the focal individual was removed by dip net, anesthetized with MS222 (tricaine methanesulfonate, Crescent Research Chemicals), and weighed. We then used a 0.3-ml syringe with a 28-gauge, 1/2 inch needle to inject the male intraperitoneally with either saline control (0.9% NaCl solution with 0.2% bovine serum albumin) or one of the following four treatments: (1) 0.1  $\mu\text{g}$  AVT (Sigma, St. Louis, MO)/g body weight; (2) 1  $\mu\text{g}$  AVT/g body weight; (3) 10  $\mu\text{g}$  AVT/g body weight; or (4) 2.5  $\mu\text{g}$  Manning compound ( $\beta$ -mercapto- $\beta$ ,  $\beta$ -cyclopentamethylenepriopionyl<sup>1</sup>, *O*-Me-Tyr<sup>2</sup>, Arg<sup>8</sup>-vasopressin; Sigma)/g body weight. Both AVT and Manning compound were suspended in a saline solution identical to the control. The doses of AVT and Manning compound used in this experiment were within the range of doses shown to alter social behaviors in other fishes (e.g., Bastian et al., 2001; Semsar and Godwin, 2004; Semsar et al., 2001). Immediately after injection, the experimental subject was placed in an aerated beaker (1 l) to recover from anesthesia (3–4 min) before being returned to the social tank. Pupfish in the tank were then allowed to reestablish a social structure (30 min). Pilot studies showed that 30 min was sufficient for males to return to their territories and for fish to return to a state similar to that before manipulation. Following this 30-min period, we recorded behavior of the focal male for another 60 min.

Given the protected status of pupfish populations in Death Valley, we minimized the number of fish used in this study by following a repeated measures experimental design. Thus, each focal male received every treatment with 4 days separating consecutive injections. The order of injections followed a balanced Latin squares design, and there were no significant carry-over effects of prior treatment for any behavior (Williams, 1949). All hormone solutions were coded, and the observer was unaware of the treatments.

### Statistical analyses

First, for the control injection, we used a one-sample *t* test to determine if there was a change in behavior between the pre- and postinjection observation periods. Next, we analyzed the effects of hormone administration as the change in the frequency of behaviors between pre- and postinjection observation periods. Specifically, we scored this change as the  $\ln[(\text{No. of postinjection behaviors} + 1) / (\text{No. of preinjection behaviors} + 1)]$ , where ‘No. of preinjection behaviors’ was the number of times a specific behavior was performed during the 60-min observation period before injection, and ‘No. of postinjection behaviors’ was the number of times the behavior was performed during the 60-min postinjection observation period. We then conducted repeated measures ANOVAs on these scores to determine whether there was a difference among the four treatments and control for each behavior (Zar, 1996). Since each male received all treatments, we next used paired *t* tests to determine whether the score for a single treatment differed from control. Due to the number of paired *t* tests performed, we Bonferroni corrected these pairwise comparisons to maintain an overall  $\alpha$  level of 0.05; differences between a treatment and the control were only considered statistically significant if  $P < 0.0125$ . We calculated *t* tests using SYSTAT 8.0 software (SPSS Inc.), and *P* values for repeated measures ANOVAs were obtained with StaTable 1.0.1 (Cytel Software Corporation). All statistical tests were two tailed.

### Experiment 2: effects of AVT on pupfish behavior in the wild

Between May 25 and May 31, 2003, we administered AVT to wild, sexually mature male pupfish ( $n = 8$ ) in the Amargosa River. Mean body weight of male pupfish was  $1.71 \pm 0.19$  g (no difference between treatments; *t* test,  $P = 0.4557$ ) and standard length was  $36.91 \pm 1.31$  mm (no difference between treatments;  $P = 0.6632$ ). Mean time of injection was similar between treatments (AVT treatment, 13:27 p.m.; saline treatment, 13:18 p.m.). Salinity during these experiments was 1.1 ppt and water temperature was  $31.3 \pm 0.85^\circ\text{C}$  (range: 25.5–36.3°C). Although the temperature of the Amargosa River fluctuates widely, there was no difference between treatments (*t* test,  $P = 0.9507$ ).

### Testing arenas

Pupfish were tested in four enclosed arenas (approximately  $1.7 \times 6$  m in dimensions, approximately  $10 \text{ m}^2$ ) constructed with wire screen (1 mm mesh) in the river. Arenas were always located against a riverbank to keep pupfish in shallow (<8 cm) water where the current is slower and males regularly establish reproductive territories. Overnight or when no experiment was being conducted, we opened the arenas so fish could move freely between the enclosure and river. Immediately before experimentation,

one of the arenas was closed to confine all fish that happened to be inside. One male was then collected from the arena, marked with a red elastomer tag (Northwest Marine Technologies, Inc., Shaw Island, WA) injected on the dorsal surface of the body to the left of the dorsal fin, and administered hormone. Following the experiment, we opened the enclosure to allow fish access to the river. On two occasions, sealing the enclosure trapped a pupfish that had been injected with hormone on a previous day—these tagged males were released to the river before beginning the experiment.

### Hormone administration and behavioral observations

Each male pupfish was collected by dip net and placed into an aerated beaker (1 l). The fish was immediately anesthetized (MS222), weighed, and measured. Males were then injected (5  $\mu\text{l}$  Hamilton syringe) intraperitoneally with either AVT (1  $\mu\text{g/g}$  body weight) or saline control (0.9% NaCl with 0.2% bovine serum albumin). Each experimental subject received only a single injection of either AVT or saline. Following hormone administration, the fish recovered from anesthetic in an aerated bucket for approximately 4–5 min before being released back into the testing arena. After a 25-min period to allow pupfish to reacclimate to the stream, an observer standing on the streambank used a tripod-mounted digital video camera (Sony DCR-TRV 19) to track and record the focal pupfish for 20 min. All videotapes were coded so that the observer was unaware of hormone treatment when later scoring behaviors.

### Statistical analysis

We used two-sample *t* tests to compare the frequency of behaviors between AVT and control treatments. In three cases (frequency of aggressive charges by the focal male, feeding bites, and resting), the data failed to conform to the assumptions of normality so we first  $\ln$ -transformed them to homogenize variances. To determine whether AVT affected how males responded to the aggressive behavior of other male pupfish, we used Mann–Whitney *U* tests to compare the proportion of responses between AVT and control groups. All statistical tests were two-tailed and performed using SYSTAT 8.0 software (SPSS Inc.).

## Results

### Experiment 1: effects of AVT and a $V_1$ antagonist

#### AVT inhibited aggression toward males and females

We found significant changes in the initiation of interactions with nips among the four treatments and control (Fig. 1A). Significant declines in nipping occurred in response to both 1 and 10  $\mu\text{g}$  AVT/g body weight. Injection

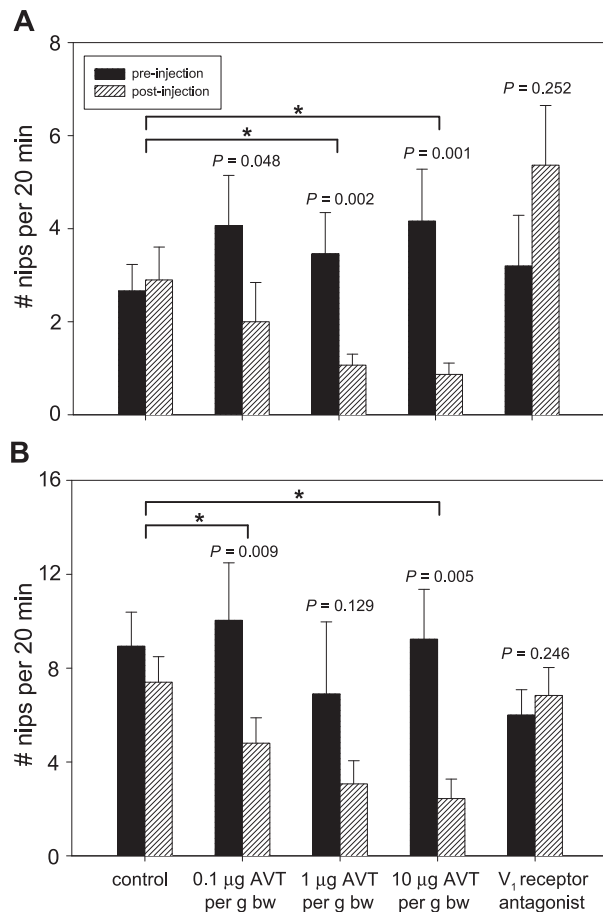


Fig. 1. Effects of AVT and the  $V_1$  receptor antagonist on the initiation of social interactions with aggressive nips directed toward males (A) and females (B) in the laboratory. Statistical comparisons examined whether hormone treatments differed from control in the change in behavior from the preinjection to postinjection observations. The treatments varied in how they affected nipping directed at males [ $F(4,36) = 6.8086, P = 0.0003$ ] and at females [ $F(4,36) = 4.010, P = 0.0086$ ].  $P$  values represent paired  $t$  tests between the change in nipping caused by that treatment and the change caused by the control, and asterisks indicate a significant difference ( $P < 0.0125$  after Bonferroni correction). Sample size is  $n = 10$  for all treatments. Values are plotted as mean  $\pm$  SE.

with 0.1 µg AVT/g body weight and Manning compound had no effect. The frequency of aggressive nipping directed at females also varied among treatments (Fig. 1B). Injection

of 0.1 and 10 µg AVT/g body weight decreased nipping, while neither 1 µg AVT/g body weight nor Manning compound had a significant effect. Injection of saline control did not alter the initiation of social interactions by aggressive nips directed at either males or females.

Vasotocin had similar effects on the initiation of social interactions by aggressive charges toward males and females (Table 2). Saline control did not alter the initiation of social interactions with aggressive charges toward males, but pairwise comparisons showed that injection of 10 µg AVT/g body weight decreased charging. Injections of 1 µg AVT/g body weight, 0.1 µg AVT/g body weight, and Manning compound had no effect. Aggressive charging at females showed a similar response, with only the 10-µg AVT/g body weight treatment inhibiting charging. Injection of saline control did not affect the frequency of aggressive displays toward males, but there was no effect of AVT or the AVP  $V_1$  antagonist on displaying.

#### AVT did not alter courtship behavior

There were no significant differences in the frequency of courtship sidles among the four treatments and control (Fig. 2). Pairwise comparisons confirm that behavioral responses to injection of 0.1, 1, 10 µg AVT/g body weight, and Manning compound did not differ from saline control. Injection of saline control did not change the frequency of courtship by males.

#### Highest dose of AVT (10 µg/g body weight) inhibited feeding

Fig. 3 shows that there were significant differences in how the four treatments and control affected the frequency of feeding bites. The 10-µg AVT/g body weight dose induced a significant decline in feeding relative to control, while injections of 0.1 µg AVT/g body weight, 1 µg AVT/g body weight, and Manning compound had no effect. Saline control injection did not cause a change in feeding between pre- and postinjection observations. Given the size of the testing tank and the hiding spots provided by rock structures, the decrease in feeding behavior from the highest AVT dose appeared to be a direct effect of the hormone

Table 2

Effects of AVT and the  $V_1$  receptor antagonist on the frequency (charges per 20 min, mean  $\pm$  SE) of social exchanges initiated with aggressive charges at males and females

		Control	0.1 µg AVT/g body weight	1 µg AVT/g body weight	10 µg AVT/g body weight	$V_1$ receptor antagonist	ANOVA
Charges at males	Preinjection	11.73 $\pm$ 2.70	13.90 $\pm$ 4.36	13.67 $\pm$ 2.30	19.37 $\pm$ 6.21	11.57 $\pm$ 2.64	$F(4,36) = 7.065$ $P = 0.0003^*$
	Postinjection	15.23 $\pm$ 3.99	13.97 $\pm$ 6.66 $P = 0.048$	7.27 $\pm$ 1.14 $P = 0.025$	6.03 $\pm$ 1.24 $P = 0.009^*$	19.50 $\pm$ 5.60 $P = 0.314$	
Charges at females	Preinjection	41.57 $\pm$ 4.51	40.20 $\pm$ 6.57	42.97 $\pm$ 5.00	45.04 $\pm$ 7.56	38.64 $\pm$ 3.97	$F(4,36) = 4.354$ $P = 0.057$
	Postinjection	45.40 $\pm$ 6.55	38.47 $\pm$ 6.06 $P = 0.268$	29.87 $\pm$ 5.75 $P = 0.065$	21.07 $\pm$ 3.53 $P = 0.005^*$	36.07 $\pm$ 5.77 $P = 0.282$	

Note.  $P$  values under treatment columns represent paired  $t$  test comparisons between the change in behavior caused by hormone injection and by saline control. For each treatment, the change in behavior was quantified as  $\ln[(\text{No. of postinjection behaviors} + 1) / (\text{No. of preinjection behaviors} + 1)]$ .

\* Indicates a significant difference ( $P < 0.0125$  following Bonferroni correction) between hormone treatment and control.

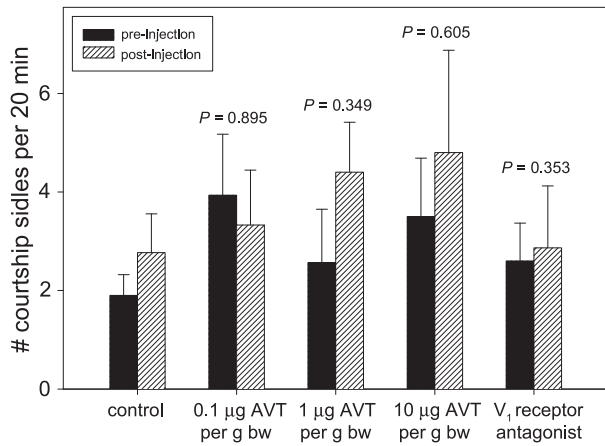


Fig. 2. Influence of AVT and the V<sub>1</sub> receptor antagonist on courtship sidling by male pupfish in the laboratory. Values are plotted as mean ± SE for pre- and postinjection observation periods. There was no difference in the change in courtship among treatments [ $F(4,36) = 1.426$ ,  $P = 0.245$ ].  $P$  values represent pairwise comparisons between the change in courtship caused by that treatment and the change caused by the saline control;  $n = 10$  for each treatment.

itself and not a by-product of being aggressively excluded from feeding opportunities by other fish.

#### Concurrent changes in aggression of nonfocal fish

Nonfocal males showed a trend toward increasing the frequency of agonistic interactions initiated with aggressive charges and nips (combined) toward focal males given increasing doses of AVT [repeated measures ANOVA,  $F(4,36) = 3.1895$ ,  $P = 0.0243$ ], although no treatment differed significantly from the control in Bonferroni-corrected pairwise comparisons. Similarly, the frequency of interactions initiated with displays from nonfocal males

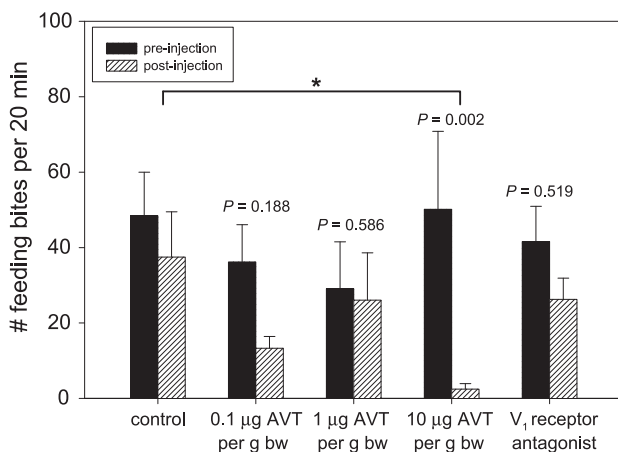


Fig. 3. The effects of AVT and the V<sub>1</sub> receptor antagonist on the frequency of feeding bites varied among treatments [ $F(4,36) = 6.644$ ,  $P = 0.0004$ ] in the laboratory experiment. Feeding frequency is shown for both the pre- and postinjection observation periods for each treatment.  $P$  values represent pairwise comparisons between the change in feeding caused by that treatment and the change caused by the saline control, and asterisks indicate a significant pairwise difference ( $P < 0.0125$  after Bonferroni correction). Values are plotted as mean ± SE, and  $n = 10$  for each treatment.

also showed a positive trend with increasing AVT doses given to the focal male [ $F(4,36) = 2.733$ ,  $P = 0.0439$ ], although there were no significant pairwise differences from control.

#### Experiment 2: effects of AVT on behavior in the wild

##### AVT reduced aggression but did not affect courtship

Effects of AVT on aggressive behaviors in wild fish were similar in some respects to results from the laboratory. In wild males, 1 µg AVT/g body weight decreased the frequency of social exchanges initiated by aggressive charges at nonfocal males (Fig. 4;  $t$  test,  $df = 14$ ,  $t = -2.235$ ,  $P = 0.0422$ ) but had no effect on the frequency of interactions initiated with either nips ( $P = 0.6387$ ) or displays ( $P = 0.2924$ ). There was no effect on charges or nips (combined) directed at females ( $P = 0.3151$ ).

AVT had no effect on either the frequency of courtship sidling (saline,  $1.25 \pm 1.11$  per 20 min; AVT,  $2.53 \pm 1.44$ ;  $t$  test,  $P = 0.4944$ ) or the mean duration of time spent sidling (saline,  $15.63 \pm 15.34$  s per 20 min; AVT,  $14.25 \pm 9.75$ ;  $P = 0.9516$ ). Similarly, there was no effect of AVT on the frequency of S-shape spawning events ( $P = 0.3919$ ). The frequency of feeding bites (saline,  $2.41 \pm 1.02$  per 20 min; AVT,  $2.16 \pm 0.45$ ;  $P = 0.5054$ ) as well as the frequency (saline,  $2.00 \pm 0.93$  rests per 20 min; AVT,  $4.38 \pm 2.34$ ;  $P = 0.8474$ ) and duration of resting (saline,  $47.6 \pm 27.7$  s per 20 min; AVT,  $58.4 \pm 28.1$ ;  $P = 0.7892$ ) by focal males were likewise unaffected by AVT.

##### Nonfocal males decreased aggression toward focal males

Overall, nonfocal males decreased the initiation of aggressive interactions with focal males, and focal males responded less aggressively to nonfocal males (Table 3). Nonfocal males charged focal males less if the focal individual had received an injection of AVT instead of

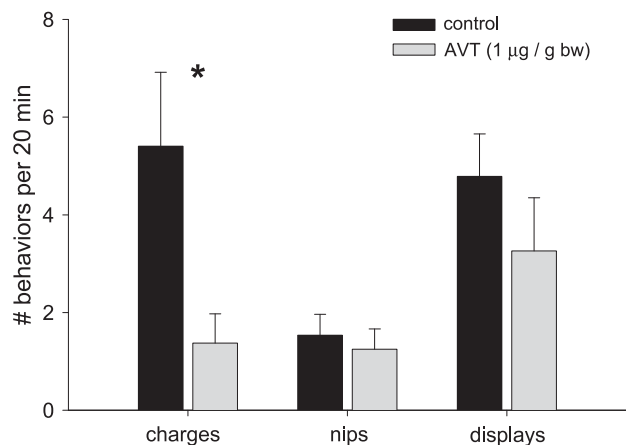


Fig. 4. Effects of AVT (1 µg/g body weight) on aggressive behaviors of wild male pupfish in the Amargosa River. AVT decreased the frequency of agonistic interactions initiated by aggressive charges toward other males ( $*t = -2.235$ ,  $P = 0.042$ ), but had no effect on interactions initiated with nips or displays. Values are plotted as mean ± SE, and  $n = 8$  for both treatments.

Table 3

Frequency (mean  $\pm$  SE) of charges and displays by wild nonfocal males toward focal males injected with either saline control or AVT (1  $\mu$ g/g body weight) and the proportion of behavioral responses by focal males to the aggression of these nonfocal fish

	Nonfocal behavior (no. per 20 min.)		Focal male behavior (proportion responses)					
	Control	AVT	Charges and nips		Displays		Retreats	
			Control	AVT	Control	AVT	Control	AVT
Charges	26.30 $\pm$ 5.58	11.26 $\pm$ 3.40	0.05 $\pm$ 0.02	0.00 $\pm$ 0.00	0.07 $\pm$ 0.04	0.05 $\pm$ 0.04	0.88 $\pm$ 0.05	0.95 $\pm$ 0.04
	$*t = -2.300, P = 0.037$		$*U = 16, P = 0.027$		$U = 26, P = 0.469$		$U = 48, P = 0.073$	
Displays	11.73 $\pm$ 1.06	9.14 $\pm$ 0.76	0.04 $\pm$ 0.02	0.02 $\pm$ 0.01	0.40 $\pm$ 0.10	0.12 $\pm$ 0.04	0.56 $\pm$ 0.11	0.86 $\pm$ 0.04
	$t = 1.981, P = 0.068$		$U = 27, P = 0.523$		$*U = 13, P = 0.044$		$*U = 50.5, P = 0.051$	

Nonfocal behaviors were analyzed with  $t$  tests, and focal male responses were analyzed with Mann-Whitney  $U$  tests.

saline. AVT-injected fish responded less aggressively to the charges of nonfocal males than did saline-injected fish, seen as a decrease in the proportion of responses as charges and nips (combined). Although there was no difference in aggressive displaying by nonfocal males toward AVT- and saline-administered fish, AVT-injected males returned fewer displays and retreated more from these social interactions.

## Discussion

### *AVT modulation of behavior*

Here we showed that exogenous administration of AVT inhibited aggression in male Amargosa River pupfish. This inhibition was seen in two behavioral contexts—as a reduction in the initiation of aggressive social interactions and as a reduction in aggressive responses to social exchanges initiated by other fish. These effects on aggression were observed both in the laboratory and in freely behaving pupfish in the wild. Administration of a  $V_1$  receptor antagonist, however, failed to significantly alter aggression in these same fish. Levels of endogenous vasotocin have never been examined in pupfish, but the high aggression of territorial male pupfish may be associated with low endogenous AVT that precludes a large change in behavior to the  $V_1$  receptor antagonist. Overall, however, our results are generally consistent with other studies in territorial fish where peripherally administered AVT reduced aggression (Bastian et al., 2001; Semsar et al., 2001).

Although we found that vasotocin inhibited aggression in male pupfish, we did not find significant changes in courtship. This result is contrary to other studies where exogenous vasotocin has been shown to affect courtship in fish. In the weakly electric fish *A. leptorhynchus*, AVT increased the production of type-I electric organ chirps, a signal emitted during courtship and mating (Bastian et al., 2001). Salek et al. (2002) also found that AVT increased courtship-attending behavior in male white perch (*Morone americana*). Given the results of these other studies, it is unclear why exogenous AVT did not modulate courtship in pupfish. One possible explanation is that pupfishes in Death Valley have evolutionarily lost the complex courtship

sequences seen in other pupfish species (Liu, 1969), which may make it difficult to detect effects of AVT on sexual behaviors. Alternatively, social groups of pupfish in our laboratory experiments contained only four females. Pupfish females continuously produce eggs when in reproductive condition, but they may only spawn a few eggs each day. Our observations of intermittent spawning indicate that experimental females were in reproductive condition, but they may not always have been receptive to males during every testing period. Still, our results in both the laboratory and in the Amargosa River are consistent since courtship was not affected in either context. Whereas AVT and its homologue AVP are by and large considered mediators of sexual behaviors, studies in birds have also found that AVT can alter aggression without affecting courtship (Goodson, 1998a,b; Goodson and Adkins-Regan, 1999; Goodson et al., 2004).

In other fishes, modifications to the vasotocin system have also been shown to affect suites of behaviors related to courtship and aggression (see Bastian et al., 2001; Semsar and Godwin, 2004; Semsar et al., 2001). While we did not specifically test this idea, AVT may affect multiple behaviors by altering the response to social stimuli and causing changes in habitat use. For instance, in bluehead wrasse, exogenous AVT caused nonterritorial terminal-phase males to reduce movement and adopt territorial-typical behaviors over locations of coral reef that were not usually used for spawning sites (Semsar et al., 2001). This shift in habitat use was associated with increases in both aggression and courtship. In our experiments with pupfish in the Amargosa River, we found that nonfocal males were less aggressive toward pupfish that had received injection of AVT. Yet in the laboratory, nonfocal males increased aggression toward AVT-injected males. We hypothesize that this discrepancy may have occurred because fish tested in the wild were free to leave shallow areas of the stream where nonfocal males defended territories, whereas males tested in the laboratory were constrained by their testing tank.

The changes in aggression that we observed in response to vasotocin may also reflect the behavioral shifts seen as pupfish switch between alternative reproductive tactics. Pupfish males exhibit alternative reproductive tactics that vary in aggression and courtship (Kodric-Brown, 1986).

These behavioral tactics are reversible, and pupfish may switch between them many times depending on the current ecological conditions (for a review, see [Watters et al., 2003](#)). Although the physiological underpinnings of such behavioral variation in pupfish are not known, changes in AVT physiology have been implicated to mediate alternative phenotypes in other fishes ([Foran and Bass, 1999](#); [Grober et al., 2002](#); [Miranda et al., 2003](#)).

#### *Mechanisms of AVT action*

In the current study, we administered AVT intraperitoneally. Such peripheral injections likely cause systemic increases in AVT levels so the site of AVT action on behavior is unclear. Receptors for AVT have been found in the brain of fish ([Moons et al., 1989](#)), and the absence of a blood-brain barrier in teleost fishes suggests that peripheral AVT could act directly on the brain to modulate behavior. Alternatively, high peripheral doses of AVT have been shown to activate the hypothalamic–pituitary–adrenal (HPA) axis and stimulate increases in plasma corticosteroids in birds (e.g., [Nephew and Romero, 2000](#)). In fishes, AVT has been shown to stimulate secretion of adrenocorticotrophin ([Baker et al., 1996](#)). The highest dose of AVT (10 µg/g body weight) that we used in the laboratory experiments caused a dramatic reduction in feeding, suggesting that the behavioral effects of this dose may have been in part caused by HPA axis activation either as a direct effect of AVT or as a by-product of the elevated aggression of nonfocal males. Nevertheless, in wild pupfish in the Amargosa River, a lower dose of AVT (1 µg/g body weight) inhibited aggression without altering either feeding or resting behaviors. This result suggests that AVT can mediate changes in aggression without suppressing all behavior nonspecifically.

Since systemic elevations in AVT can have behavioral effects, future work should examine how natural fluctuations in plasma AVT relate to behavior. In rainbow trout (*Oncorhynchus mykiss*), levels of plasma AVT follow a diel cycle with lowest and highest levels occurring, respectively, at sunrise and sunset ([Kulczykowska, 1999](#)). AVT mRNA levels in parvocellular neurons of this species appear to follow a similar cycle ([Gilchrist et al., 1998](#)). Wild pupfish in the Amargosa River show daily changes in activity with low aggression after sunrise and before sunset and increased aggression during midday. These behavioral changes are likely regulated in part by water temperature, but endogenous AVT cycles could also play a role.

#### *AVT/AVP and behavioral evolution*

Accumulating evidence from studies that have examined how AVT or AVP influences behavior suggests that these hormone systems may underlie differences in social behaviors among taxa (for a review, see [Insel and Young, 2000](#)). For instance, in two avian species with a territorial

social organization—the field sparrow (*Spizella pusilla*) and violet-eared waxbill (*Uraeginthus granatina*)—infusion of AVT into the septum inhibited aggression in males ([Goodson, 1998a,b](#)). Yet in the colonial zebra finch (*Taeniopygia guttata*), AVT facilitated aggression in males ([Goodson and Adkins-Regan, 1999](#); [Goodson et al., 2004](#)). Likewise, [Insel et al. \(1994\)](#) found a relationship between the expression pattern of vasopressin receptors in the brain and variation in social organization among species of voles. Such studies indicate that components of AVT physiology can be shaped by the ecological conditions that animals experience and suggest that changes in AVT or AVP physiology may be one proximate mechanism involved in the diversification of social behaviors. Still, comparative studies on AVT or AVP and behavior are scarce, and the majority of this work has compared taxa that diverged millions of years ago, thereby making it difficult to address the process of how social behaviors evolve.

More recent work, however, indicates that similar changes to AVT/AVP physiology might underlie behavioral differences among populations that have been isolated for a much shorter period of time. For instance, [Cushing et al. \(2001\)](#) found that vasopressin had different effects on affiliation in prairie voles (*Microtus ochrogaster*) from two populations that vary in social organization. In Amargosa pupfish, our own work has shown that fish from the Amargosa River differed in the size of preoptic AVT-immunoreactive neurons when compared to same-sex individuals from a closely related population that has been separated for only 400–4000 years ([Lema and Nevitt, 2004](#)). Although these differences in AVT cell size could represent a plastic neuroendocrine response to dissimilar ecological conditions, males from these populations also differ in social behaviors with males behaving more aggressively in the population with smaller AVT-immunoreactive neurons. The population differences in neural AVT phenotypes combined with our current finding that exogenous AVT can modulate aggression in male pupfish suggest that the differences in brain AVT may be related to the behavioral differences between these populations. Similar variation in the size of magnocellular AVT-ir neurons has recently been found among males from populations of guppies (*Poecilia reticulata*) in Trinidad ([Godwin et al., 2003](#)), and this variation could be related to behavioral differences among these populations as well (e.g., [Rodd and Sokolowski, 1995](#)).

Nevertheless, how AVT physiology relates to behavioral variation remains largely unclear. Exogenous AVT can have different behavioral effects depending on the species, but it is rarely known physiologically why these differences exist or how they relate to ecological differences between taxa. Environmental conditions influence social behaviors in many animals including pupfish (for a review, see [Watters et al., 2003](#)), and some environmental factors are known to directly impact the vasotocin system (i.e., salinity; [Warne, 2002](#)). Understanding how environmental conditions shape



both AVT physiology and behavior concurrently will inform how differences in AVT neural phenotypes arise, as well as provide a clearer picture of the role for AVT in the evolution of behavior.

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