Arginine vasotocin activates aggressive calls during paternal care in the Puerto Rican coquí frog, *Eleutherodactylus coqui*

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**HIGHLIGHTS**

- AVT-injected males showed a dramatic and significant increase in aggressive calls.
- Vocalization that AVT activates depends on behavioral phenotype of the male.
- Vocalization that AVT activates also depends on the social and environmental context.
- AVT did not significantly elevate paternal males to territorial status.
- AVT nor did it significantly induce abandonment of eggs/embryos.

**ABSTRACT**

The Puerto Rican coquí frog, *Eleutherodactylus coqui*, is a directly developing frog that exhibits male territoriality and paternal behaviors. Male frogs also produce advertisement and aggressive vocalizations or calls. Territorial males emit advertisement calls to delineate territories and attract mates. Paternal males guard and brood the directly developing embryos during embryogenesis and up to five days after hatching; advertisement calling is normally absent or infrequent during paternal care. Territorial and paternal males commonly produce aggressive calls during agonistic situations. The neuropeptide, arginine vasotocin (AVT), has been shown to promote calling in anurans, including *E. coqui*. The objective of this study was to determine if exogenous AVT promotes calling and territorial behavior in paternal males and if it promotes males to abandon their offspring. Injections (IP) of AVT were given to paternal males immediately before the scotoperiod. Frogs were monitored for at least four hours after the injection and the following morning for calling activity and abandonment of egg clutches. AVT-injected males showed a dramatic and significant increase in aggressive calls compared to control males (saline injections). Exogenous AVT did induce advertisement calling in some paternal males but did not significantly elevate paternal males to territorial status nor did it significantly induce abandonment of eggs/embryos. In conclusion, the type of vocalization that AVT activates in *E. coqui* depends upon the reproductive state of the male and the social environment that surrounds the male.

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1. Introduction

Vertebrates display a remarkably rich and diverse array of parental care. Maternal and biparental care occurs in many species of birds and mammals, whereas exclusive paternal care is relatively more common in fish and amphibians [31]. Indeed, approximately 10% of all frog species exhibit some form of parental care including maternal, biparental, and paternal [1,13,30]. Interestingly, in the species (400+) Neotropical frog genus, *Eleutherodactylus*, parental care can be paternal, maternal, amphisexual (where either the male or the female attends to egg clutches), or nonexistent [25]. Furthermore, the tadpole stage has been eliminated in this genus and frog embryos undergo direct development in clutches of terrestrial eggs and hatch as small neonates in the adult phenotype [30]. The Puerto Rican coquí frog, *Eleutherodactylus coqui*, is a terrestrial amphibian that exhibits territorial behavior and provides paternal care for its directly developing young [27].

The neuropeptide, arginine vasotocin (AVT), and its mammalian homologue, arginine vasopressin (AVP), are essential to many social and reproductive behaviors [4,9,8,7]. AVT plays a modulatory role in amphibian reproduction by promoting territorial, mating, and courtship behaviors [4,15,14]. In anurans, AVT promotes male advertisement calling and reproductive behaviors. Specifically, it
has been shown to increase the frequency and the effectiveness of advertisement calling of bullfrogs [3], to stimulate calling behavior in cricket frogs [6] and green treefrogs [5], and to increase male social status and calling-site acquisition in gray treefrogs [20]. It has been reported in male túngara frogs that AVT alters mating calls and decreases call attractive to female frogs [10,11]. In E. coqui, exogenous injections of AVT induced advertisement calling in both non-calling (non-territorial) and calling (territorial) males and promoted the non-territorial males to move into adjacent areas and initiate calling in their new territories [23].

Male E. coqui communicate with conspecifics primarily through vocalizations. The advertisement call of E. coqui consists of two notes: a low-pitched “co” note emitted for territorial defense, followed by a higher-pitched “qui” note for mate attraction [16,17]. Resident male E. coqui maintain and defend territories that are delineated by acoustical properties, call frequency, length, and intensity, all of which vary depending on calling site properties such as vegetative cover [18]. Aggressive (or encounter calls) differ significantly from advertisement calls [21,29,30]. The aggressive call typically consists of one (or sometimes two) “co” notes followed by series of “qui” notes repeated in rapid succession. Aggressive calls have been reported to be utilized in aggressive or defensive engagements with conspecifics [21,27].

E. coqui have three different male behavioral modes, territorial (calling), non-calling, and paternal. Theoretically, any reproductively capable male can be any mode. For example, territorial males may call for a portion of the night or for a series of successive nights only to become non-calling for a period of time (personal observations). Territorial males adopt dominant male postural characteristics (elevated torso, erect body, inflated gular region) and emit nocturnal advertisement calls from elevated sites to delineate territories and attract females. Non-calling or non-territorial males maintain submissive postural characteristics (dorsoventrally flattened torso, ventrum contacting or bordering substrate) within the territories of calling males and typically do not call. Aggressive encounters between two males can be accompanied by aggressive calls and may escalate into overt physical combat bouts if one of the males does not submit [27]. Following mating (paternal) males provide exclusive parental care and remain with their eggs during embryological development and up to five days after hatching. Paternal care involves both brooding the eggs and actively defending them against predators. Advertisement calling is typically reduced or absent during paternal care. Further, if the male is significantly stressed or threatened, he may cannibalize or abandon his eggs [27]. The objective of this study was to determine if exogenous AVT administration could activate advertisement calling, initiate territorial behavior, and consequentially promote offspring abandonment in paternal males in a semi-natural setting. It was hypothesized that AVT administration would activate territorial advertisement calling and behavior, and paternal males would abandon their offspring. Alternatively, it was hypothesized that AVT would activate aggressive calling in paternal males due to the fact that they are defending their clutches and the role of AVT in aggressive behavior.

2. Materials and methods

Adult E. coqui utilized in this study were wild-caught frogs from the Caribbean National Forest of northeastern Puerto Rico. They were captured under permits issued by the Departamento de Recursos Naturales, Puerto Rico. They were maintained in the animal care unit in the Department of Psychology at the University of Michigan. Care of all frogs was conducted in accordance with the regulations of the University of Michigan UCUA. Adult E. coqui were housed in semi-natural (3 males and 3 females per tank) 50 gallon glass terraria (27 cm × 27 cm × 106 cm) with sphagnum moss flooring and several water dishes; frogs were fed live crickets. Eight 1” (2.54 cm) PVC pipes (16 cm length) were distributed along the edges of the tanks for shelter, mating, and nest sites. Frogs were allowed to acclimate for several weeks until spontaneous mating occurred.

Paternal males found guarding egg clutches were removed from their eggs with minimal disturbance and injected (IP) with 50 μg AVT in 100 μl of 9% saline (NaCl) solution. The dose was determined from previous AVT investigations on E. coqui [23]. Control animals were given injections (IP) of 100 μl 5% saline. Frogs were injected immediately prior to the beginning of the scotoperiod, marked with non-toxic fluorescent pigment powder (Peart Ex Pigments; Rupert, Gibbon, Spider Inc., Healdsburg, CA) for individual recognition, placed back on their eggs, and were continually monitored for up to four hours after the injection and the following morning. Only one frog/terrarium was ever tested during the investigational time period and no individual frog was tested repeatedly in either the experimental or control groups. Only one paternal male was present in the terrarium during testing. All aspects of frog behavior including, calling activity, male–male or male–female interactions, and nest fidelity, were recorded. Observations were made using a red-light headlamp. Eggs clutches were declared abandoned if the paternal male was not back on the eggs the following day after treatment and remained off the eggs permanently.

Following the observational period, eggs from the paternal male were staged according to the Townsend and Stewart [26] embryonic staging protocol for E. coqui (stages TS 1–15). Eggs/embryos were clustered into three groups based on developmental stage: early (stages TS 1–5), mid (TS stages 6–10), and late (TS stages 11–15) embryonic ages. Control animals that were given saline injections underwent the same experimental procedure. Thirty-six paternal males were injected with AVT and twenty-five controls were injected with saline (Table 1). Behavioral differences between AVT-injected and saline-injected paternal males were determined using two-tailed Fisher’s exact test.

3. Results

Paternal males injected with AVT emitted a significantly (P = 0.0001) higher number of aggressive calls than saline-injected males (Fig. 1). Twenty-six (72.2%) of 36 AVT-injected males emitted aggressive calls (11/14 early, 13/14 mid, 2/8 late embryonic groups) and only two (8.0%) of 25 saline-injected controls produced aggressive calls (2/15 early, 0/6 mid, 0/4 late) (Table 1). These calls were emitted while the males were directly on top of their egg clutches, on their PVC tube, or on the tank floor adjacent to their eggs.

The total number of AVT-injected paternal males had a significantly (P = 0.035) higher number of advertisement calls than saline-injected males (Fig. 2). Seven (19.4%) of 36 AVT-injected males emitted advertisement calls during the observational period (3/14 early, 3/14 mid, 1/8 late) (Table 1). Control males did not emit advertisement calls. All of the AVT-injected males that emitted advertisement calls also emitted aggressive calls during the observational period. Although both aggressive and advertisement calls were significantly higher in AVT-injected males, there was a significant difference between numbers of aggressive calls (N = 26) and the number of advertisement calls (N = 7) emitted by AVT-injected paternal males (P = 0.0001).

Egg abandonment did not significantly (P = 0.35) differ from AVT-injected paternal males compared to saline-injected controls (Fig. 3). Thirty of 36 (83.3%) AVT-injected males (10/14 early, 12/14 mid, 8/8 late) and 18 of 25 (72.0%) control males (9/15 early, 5/6 mid, and 4/4 late) were on their eggs the morning after the injection (Table 1). Paternal males of younger clutches had a higher
rate of abandonment than older clutches in both the control and AVT conditions (Fig. 3). However, this higher rate was not significantly \((P > .05)\) different across the experimental conditions. In three of the 13 cases of abandonment, the eggs were canni-
balized by another frog during the night time period, after the
observational time and prior to the morning check on the eggs and
male. Seven AVT-paternal males emit advertisement calls and six
AVT-paternal males abandoned their eggs. However, only two AVT-
paternal males \((P > .05)\) did both, emitted advertisement calls and
subsequently abandoned their clutch to become territorial males
(Table 1).

Upon injection, there was a short recovery period characterized
by low activity \((mean \pm 20 \pm 9.7 \text{ min})\) in which the paternal male
recovered from the handling and injection. Upon recovering, all
AVT-injected males adopted erect body/torso-elevated postures
on either the terrarium floor or on top of PVC pipes. None of
the control males adopted erect postures but displayed dorsoventrally
flattened postures. AVT-injected males exhibited increased arousal
and activity levels, moving back and forth around their tube and
their eggs. Interactions of AVT-injected paternal males with other
males in their terrarium were infrequent. Conspecific males

generally ignored the AVT-activated paternal males if AVT males did not
initiate calling near them. In one intraspecific interaction, an AVT-
activated paternal male emitted aggressive calls near a calling male.
The resident male lunged at the AVT-injected male and captured
a large part of his head in its mouth for a 45 min sustained bite. The
paternal male lost the contest to the resident male and retreated
back inside the PVC pipe and remained on his eggs. Interactions
of AVT-injected and control males with females in the terrarium
were also infrequent. Two interactions occurred \((one \text{ AVT and one}
control), both of which were initiated by the female, with the paternal
male retreating back to his clutch; neither female ate the eggs.
No behavioral differences were observed or noted between AVT
and control males with regard to interaction with other males or
females in the terraria.

### Table 1

Summary of male calling, number of clutches, egg fidelity, and number of males that emitted advertisement calls and abandoned their eggs. Table includes the three sets of egg/embryo stages in both the experimental and control conditions.

<table>
<thead>
<tr>
<th>Egg stage</th>
<th>Aggressive calling</th>
<th>Advertisement calling</th>
<th>Number of clutches</th>
<th>Males returning to eggs (egg fidelity)</th>
<th># of males advertisement calling and abandon eggs (territorial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–5 (early)</td>
<td>11</td>
<td>3</td>
<td>14</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>6–10 (mid)</td>
<td>13</td>
<td>3</td>
<td>14</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>11–15 (late)</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>All (total)</td>
<td>26</td>
<td>7</td>
<td>36</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>Saline–controls</td>
<td>2</td>
<td>0</td>
<td>15</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>1–5 (early)</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6–10 (mid)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>11–15 (late)</td>
<td>2</td>
<td>0</td>
<td>25</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>All (total)</td>
<td>2</td>
<td>0</td>
<td>15</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. AVT-injected paternal males emitted significantly \((P < .00001)\) more aggressive calls than those in the control condition. AVT-injected paternal males with more late stage clutches emitted encounter calls than those with early and mid-staged eggs.

Fig. 2. There was a significant \((P < .035)\) difference in advertisement calling between AVT-injected and saline-injected paternal males. AVT administration elicited advertisement calling from paternal males brooding and guarding across all embryological stages. No control male emitted any advertisement calls.
4. Discussion

Past studies in frogs have shown that exogenously administered AVT promotes advertisement calling [5,3,6,10,12,20,23,24]. Interestingly, exogenous injections of AVT in parental E. coqui produced a significant increase in aggressive calls. Typically, E. coqui emit aggressive calls when defending their egg/embryo nest clutches from potential predators both heterospecific and conspecific [21,27]. They will also emit aggressive calls from their retreats just prior to becoming inactive either during the night or at dawn and also when two males emerge from their adjacent retreats at dusk [22]. Aggressive calling in E. coqui, at least with regard to defending their eggs/embryos, may be more defensive in origin rather than aggressive due to the fact that the paternal male is actively guarding and defending his clutch. It is established that the neural mechanisms of defensive behavior and those of aggressive behavior are separate neural pathways in mammals [2], perhaps similar and separate system is also found in anurans. Along with reproductive and territorial behavior, perhaps AVT is also important in activating neuroendocrine pathways of defensive parental behavior.

Administration of exogenous AVT to paternal male E. coqui also resulted in a smaller proportion (21.6%) but statistically significant increase in advertisement calling over control males. However, there was a significant difference between numbers of aggressive calls (N = 26) and the number of advertisement calls (N = 7) emitted by AVT-injected paternal males. Further, the percentage of paternal males emitting advertisement calls (21.6%) in response to AVT was dramatically less than the percentage of AVT-activated satellite (80%) and territorial (86%) males emitting advertisement calls from a previous study on E. coqui [23]. In that study, territorial and satellite (non-calling), essentially non-paternal male E. coqui, displayed a significant increase in advertisement calling but no increase in aggressive calls was observed in either territorial or satellite males.

Aggressive and advertisement calling are two different acoustic signals that respond to different stimuli depending on the behavioral and environment context. Typically paternal males emit few advertisement calls while brooding and guarding their clutch, indeed less than territorial males, but they are certainly capable; they also emit aggressive calls during paternity [27]. Clearly, AVT activates social and reproductive behaviors, including advertisement calling in territorial and non-calling males [23]. It appears that in paternal males, as contrast to territorial or non-calling males, AVT physiologically modulates or switches the neural output in vocalizations from advertisement calling to aggressive calls. However, in this situation, whether defensive aggressive calls or offensive advertisement calls, both signals share a common AVT neuroendocrine link.

AVT-injected males did not have a significantly higher incident of egg abandonment than control males in any of the three TS stage groupings. There was a numerical difference in clutch abandonment in the stage classes and as egg clutches increased in age, a greater percentage of the males returned to them after treatment in both the AVT and control conditions. This increase in nest fidelity as a function of egg/embryo development is evolutionarily significant. As the eggs advance in age, the male has invested more time and energy into them, consequently, a successful hatching becomes increasingly beneficial for the paternal male [28]. Townsend et al. [27] also found that egg abandonment was highest during the early developmental stages when paternal males were handled and manipulated. Males that are disturbed (stressed) can and will abandon their eggs, as well as eating their own eggs (filial cannibalism) [27]. This event occurred in both AVT and control groups but actual consumption of eggs was not directly observed, just the missing egg clutch the next morning. It is noteworthy that AVT-activated paternal males do not display any decrease in egg fidelity and any change in behavioral phenotype.

AVT stimulates advertisement calling and territorial behavior in male E. coqui but it also stimulates aggressive calls in paternal males. Boyd [4] stated that the effects of AVT on aggression and other male behaviors in frogs may vary with the species and/or the social context. Sensmar and Godwin [19] also found in bluehead wrasses (Thalassoma bifasciatum), a sex-changing fish, that the difference in responsiveness to AVT treatment among behavioral phenotypes appears to be mediated by the social context. Similarly, for male E. coqui calling, AVT function in aggressive calling in paternal males may possess different but related neuronal components that occur in advertisement calling of territorial males. It appears that the function or effect of AVT is critical for both advertisement calls in territorial acquisition and aggressive calls emitted during the defense of eggs but that this function is dependent on the social context of the male. Although specific neural mechanisms of AVT for both types of calls in E. coqui remain unknown, it is consistent with other vertebrates that the effect of AVT differs in male phenotypes and their respected social environments. In conclusion, exogenous AVT significantly activated aggressive calling in paternal males but did not significantly cause them to abandon their clutches and promote them to territorial status. In E. coqui, the physiological action of AVT depends on the social status and reproductive state of male frogs.

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