Arginine vasotocin inhibits social interactions and enhances essential activities in male common lizards (*Zootoca vivipara*)

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Running title: AVT, social behavior and physiology

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ABSTRACT

Arginine vasotocin (AVT) is known to play an important role in the regulation of social behavior in a number of vertebrate species. Nevertheless, the relationship between AVT and intraspecific interactions appears complex and in some cases contradictory. Moreover, AVT influences other behaviors, which are not primarily social including exploratory behavior, locomotion and thermoregulation. Some of these behavioral effects may be side-effects from a general influence of AVT on physiology. Indeed AVT can regulate metabolism and osmoregulation. Because most studies have been conducted using mammals and birds, its role in modulating behavior in other vertebrate groups is largely unknown. In this study we examined the effect of AVT on the social behavior of male common lizards, *Zootoca vivipara*. Moreover, considering the variety of pathways AVT could be involved in, we investigated its consequences on thermoregulatory behavior and physiological performance. In mid-June 2010, 74 males were captured from field sites (Mont-Lozère, South-eastern France) and kept in the laboratory for three weeks to obtain behavioral (reaction to conspecific odors, thermoregulation) and physiological (endurance, testosterone level) measurements. We demonstrated that injection of AVT reduced testosterone level and affected social behavior in different ways depending on the size of an individual. Specifically, small males injected with AVT were less attracted by conspecific odors than small control males, and no effect was detected in large males. Moreover, AVT promoted thermoregulatory behavior and enhanced endurance. These results are concordant with previous results obtained in this species in studies on stress suggesting that AVT may act through its influence on corticosterone secretion.

Keywords: AVT, endurance, thermoregulation, social interaction, testosterone, reptile, stress

47 **INTRODUCTION**

49 Arginine vasotocin (AVT), the non-mammalian homologue of arginine vasopressin (AVP), has been
50 shown to be involved in diverse physiological functions and behavior (Choleris et al., 2013; Sinervo
51 and Miles, 2011). In a number of vertebrate species, AVT modulates a wide variety of social and
52 reproductive behaviors, such as vocal communication, social recognition and aggressive behavior
53 (Albers, 2012; Choleris et al., 2013; Goodson and Adkins, 1999; Goodson, 1998; Kabelik et al.,
54 2013; Kagawa et al., 2013; Semsar et al., 2001; Soares et al., 2012). For example, AVT
55 administration enhances courtship and mating behaviors in male rough-skinned newts, *Taricha*
56 *granulosa* (Moore and Miller, 1983) and in male Japanese quail, *Coturnix japonica* (Castagna et al.,
57 1998). AVP has been shown to facilitate scent marking behavior in hamsters (Ferris et al., 1997).
58 However, the link between AVT and social behavior may be complex, context specific and
59 occasionally contradictory, because it varies considerably among species and social systems and
60 depends also on the site of injection (systemic or in the brain). For example, in birds AVT
61 stimulates aggression in colonial species (Goodson and Adkins, 1999), but inhibits aggression in
62 territorial species (Goodson, 1998). Concordant results are also observed in voles in which the
63 effects of AVT on aggressive behavior depend on social organization (Young, 1999). In rats, AVP
64 injected intra-peritoneally inhibits aggression (Brown and King, 1984) but in hamsters it increases
65 aggression when injection into the anterior hypothalamus (Ferris et al., 1997). Moreover, Moore
66 and Miller (1983) show in male rough-skinned newts that intracerebroventricular injections of AVT
67 are 1000 times more potent in mating behaviors than intra-peritoneal injections. In addition to its
68 role in the regulation of the social behavior, AVT and/or AVP influences other behaviors that are
69 not primarily linked to sociality including exploratory behavior in rodents (Hoglund and Meyerson,
70 1982; Skopkova et al., 1987), locomotion and calling behaviors in amphibians (Boyd, 1991) and
71 thermoregulation (Ladyman et al., 2006). Some of these behavioral effects may be derived from

more general physiological AVT function. Indeed, the influence of AVT on the regulation of water balance is well documented (Bradshaw and Bradshaw, 1996; Bradshaw and Bradshaw, 2002; Cartledge et al., 2008; Kagawa et al., 2013; McCormick and Bradshaw, 2006). For example, AVT is widely recognized as an antidiuretic hormone in reptiles (Bradshaw and Bradshaw, 1996; Butler and Snitman, 2005). Elevated levels of this hormone, stimulated by desiccation or electrolyte loading, are most likely to be responsible for renal modifications aiding water conservation but they may have an additional effect through behavioral modifications that further reduce water loss. Exogenous AVT can decrease behavioral thermoregulation in the agamid lizard *Ctenophorus ornatus* (Bradshaw et al., 2007). In addition, Ladyman et al. (2006) reported that snakes, *Notechis scutatus*, receiving injections of exogenous AVT significantly decreased their preferred body temperature. Regulation of body temperature in ectotherms is a priority and a key principal determinant of behavior (Huey, 1982). Specific changes in thermoregulatory patterns may therefore represent behavioral adjustments that help reptiles to decrease water loss and maintain homeostasis (Ladyman et al., 2006).

Because AVT is more extensively associated with male than female behaviors and appears to also be modulated by testosterone (Goodson and Bass, 2001), we investigated the consequences of an injection of AVT on the social behavior on male lizards, *Zootoca vivipara*. Few studies have focused on the social influence of AVT in reptiles (Sinervo and Miles, 2011). One source of influence of AVT on social behavior may involve the interaction between AVT and Corticosterone (Dunham and Wilcynski, 2014). Moreover, considering the diverse pathways AVT may act, we investigated the consequences of AVT treatment on thermoregulatory behavior and on physiological performance. For the latter we examined locomotor performance and testosterone levels.

94

95 **MATERIALS AND METHODS**

96

97 *Species, capture and rearing conditions*

98 The European common lizard (*Zootoca vivipara*) is a small-bodied (~5g) species in the
99 family Lacertidae that is widely distributed across Europe and Asia. We obtained lizards from field
100 sites located on Mont-Lozère (Massif Central, South-eastern France, 44° 30' N, 3° 45' E). Males
101 emerge from hibernation in mid-April, followed by yearlings and females in mid-May. Mating
102 occurs soon after the emergence of females. In mid-June 2010, we captured 74 males and housed
103 them in the laboratory for three weeks. We measured the snout-vent length (SVL) and weight of
104 each individual immediately after capture from the field. Males were maintained in individual
105 terraria (18x12x12 cm) with damp soil, a shelter and opportunities for thermoregulation. We used
106 incandescent light bulbs (25 W) for 6 hours a day from 09:00 to 12:00 and from 14:00 to 17:00 h
107 local time that created a thermal gradient ranging from 20° to 30°C. We selected these temperatures,
108 because they spanned the thermal breadth of the common lizard (van Damme et al., 1986). Males
109 were also provided water ad libitum and *Pyrallis* larvae every 5 days. Previous studies have also
110 shown that one larva every 5 days fulfills the energetic demands of this species (Massot and
111 Clobert, 1995). The rearing room was subjected to the natural photoperiod and maintained at 25°C
112 during the day and 18°C during the night. The authors attest adherence to the National Institutes of
113 Health Guide for Care and Use of Laboratory Animals.

114

115 *AVT treatment*

116 Circulating levels of arginine vasotocin were manipulated by an injection of arginine
117 vasotocin (AVT, Sigma-Aldrich, V0130). Males were randomly allocated to each treatment group:
118 AVT-injected males ($n = 37$) and PBS-injected males as control ($n = 37$). Four days after capture,
119 we injected intraperitoneally AVT treated males with 40 μ L of a solution at 0.02 μ g/ μ L AVT diluted
120 in sterile phosphate-buffered saline (PBS, Sigma-Aldrich, reference D 5773) and control males with
121 40 μ L of PBS. This concentration is similar to other studies in reptiles (Bradshaw et al., 2007;
122 Dunham and Wilczynski, 2014). Males of these two experimental groups did not differ in SVL
123 ($F_{1,73}=0.75$, $p=0.38$), or body mass ($F_{1,73}=0.22$, $p=0.63$) at the beginning of the experiment.

124

125 *Behavioral measurements: Thermoregulatory and response to conspecific odors*

126 We estimated thermoregulatory behavior of the 74 males using focal observation
127 measurements one time per day, between 9:30 a.m. until 11:45 a.m. and between 2:15 p.m. and 5:00
128 p.m. depending the behavioral sessions during 5 days (day 4 to day 9). We included time of the
129 behavioral measurements in the statistical analysis, but it was not significant. We used a blind
130 sampling procedure for obtaining thermoregulatory behavior. An observer noted the location and
131 behavior of each lizard in the terrarium without information regarding the treatment. If a lizard was
132 visible (i.e. at least a part the head was out of the shelter), the observer distinguished between two
133 behaviors: basking below the light and/or on the shelter, or non-basking, i.e. not under the light (see
134 Lecomte et al., 1993 for a detailed review of thermoregulatory behaviors). Lizards did not appear to
135 change their behavior when an observer entered the room. We summed the number of times a lizard
136 showed a given behavior (basking and non-basking) and expressed thermoregulatory behavior as
137 the proportion of basking behavior compared with total observations. This method has been
138 validated in previous studies in this species (Cote et al., 2010; Meylan et al., 2009).

139

140 To investigate the reaction of male to the presence of conspecific odors, at day 7, 20 males
141 (9 AVT treated males and 11 control males) were placed individually in a new experimental cage
142 where three pieces of blotting paper either impregnated with the odor of conspecifics or with no
143 odor were introduced. To minimize the effects of handling, we recorded behaviors after 10 min of
144 acclimation during which males can explore the terrarium (time of acclimation validated in de
145 Fraipont et al. 2000 and Meylan et al. 2009). Two trials were performed per male in the same
146 sequence, with one day between tests: (1) all three pieces with no odor as the control; (2) all three
147 pieces with the odors of two males. For each trial, we recorded time spent by males on the blotting
148 paper during 10 min. We calculated an index of attraction and avoidance called “response to
149 conspecific odors” using the time spent on blotting paper when impregnated with odors minus time

150 spent on the blotting paper without odor. To obtain odors, blotting paper was placed so that it
151 covered the entire floor of the captivity cages of donor males (two adult males) for 2 days. After the
152 scent-marking process, we cut the paper into pieces. We wore latex gloves to avoid contaminating
153 the samples. We chose odor cues because in reptiles, chemical senses play an important role in
154 intraspecific communication (Mason, 1992) and the available literature indicates that lacertids are
155 capable of finer chemosensory discrimination than was previously recognized (Aragon et al., 2001;
156 Aragon et al., 2006; Léna and de Fraipont, 1998).

157

158 *Endurance measurement*

159 We measured the endurance of male lizards two days after AVT injection (day 6). We included 22
160 AVT treated males and 28 control males in this part of the experiment. Lizards were held at a
161 temperature of 28°C, which is the mean field active body temperature of *Z. vivipara* (van Damme et
162 al., 1990), for a period of 30 min. Then we induced the males to run on a motorized treadmill (0.5
163 km/h) by gently tapping on the hind leg (Garland and Losos, 1994; Miles et al., 2007). The time
164 lizards maintained their position on the treadmill until exhaustion was our estimate of endurance.
165 Exhaustion of the individual was estimated by the loss of a righting response after 3 consecutive
166 taps on the leg.

167

168 *Testosterone level*

169 Blood samples (40–80 µl whole blood) were taken at the end of the experiment (day 10)
170 from the post-orbital sinus using 2–3 50 µl microhematocrit tubes (29 AVT treated males and 28
171 control males). Microhematocrit tubes were transferred to Eppendorf tubes that were centrifuged in
172 order to separate the plasma and subsequently stored in a freezer at – 20°C until analyzed.
173 Testosterone levels were determined using enzyme immunoassay (EIA) kits (Cayman Chemical
174 Company, Michigan, USA). The detection limit is approximately 6pg/ml (intra-assay variability
175 6.6%, inter-assay variability 8.5%).

176

177 *Data analyses*

178 We tested for the effects of AVT treatment on thermoregulatory behavior, response to conspecific
179 odors and endurance and testosterone levels. The full model contained the AVT treatment and a
180 covariate (SVL) and their interaction. We used a generalized linear model to assess the influence of
181 AVT on the response variables (GLM procedure of SAS, SAS Institute version 9.1). A minimum
182 adequate model was obtained by backward elimination of nonsignificant terms. Estimates are
183 provided with standard errors.

184

185 **RESULTS**

186 *AVT and response to conspecific odors*

187 The response to the presence of conspecific odors was explained by the interaction between
188 AVT treatment and SVL ($F_{1,16}=4.99$, $p=0.04$). Small males of the AVT group avoided the
189 conspecific odors more than small control males whereas there is no difference between
190 experimental groups for larger males (figure 1). Moreover, AVT injection had a negative effect on
191 testosterone levels ($F_{1,55}=5.05$, $p=0.03$; AVT group : 8.14 ± 1.13 ng/ml ; control group : $11.92 \pm$
192 1.22 ng/ml).

193

194 *AVT, thermoregulation and endurance*

195 The injection of AVT had significant effects on thermoregulatory behavior and endurance.
196 Indeed, the proportion of time spent basking was higher in AVT males compared to control males
197 (57.29 ± 4.4 % versus $39.45 \pm 4.4\%$; $F_{1,71}=6.19$, $p=0.01$). Basking behavior was also significantly
198 positively related to male SVL ($F_{1,71}=9.62$, $p=0.002$). In addition, time spent running was higher for
199 males in the AVT group than control males (568.04 ± 52.56 seconds versus 330.21 ± 42.02 seconds,
200 $F_{1,48}=12.80$, $p=0.0008$).

201

202 **DISCUSSION**

203 We investigated the influence of AVT on both behavioral and physiological components in *Zootoca*
204 *vivipara*. Our study yields three major results. First, we demonstrated that injection of AVT affected
205 a proxy of social behavior in different ways depending on the individual size. Specifically, small
206 males injected with AVT were less attracted by conspecific odors than small control males, whereas
207 there is no effect of the treatment for larger males. Second, AVT injection lowered testosterone
208 levels. Finally, AVT promoted thermoregulatory behavior and enhanced endurance. Moreover, these
209 effects are observed several days after the injection, while the majority of studies examine short
210 term effects (Choleris et al., 2013 for review).

211

212 *AVT and the regulation of social interactions*

213 Costs induced by competition among conspecifics have favored the evolution of avoidance
214 mechanisms such as spatial or temporal segregation (Schoener, 1974). However, the presence of
215 conspecifics may also indicate the suitability of the habitat in terms of food availability,
216 reproductive success, and predator abundance (Danchin et al., 2004; Parejo et al., 2007). As a
217 consequence, depending on the species and individuals characteristics, individuals might avoid or
218 be attracted to the presence of conspecifics. Previous work suggests that AVT could be an important
219 hormone that regulates aspects of social behavior, especially in males (Balment et al., 2006;
220 Dunham and Wilczynski, 2014; Goodson, 1998; Kabelik et al., 2009; Santangelo and Bass, 2006;
221 Semsar et al., 2001, Sinervo and Miles 2011). But the influence of this hormone is context
222 dependent. In the present study, we confirm a complex action of this hormone on the social
223 behavior of male common lizards. The influence of AVT on the response to conspecific odors
224 depended on the individual body size. More precisely, small AVT-injected males were less attracted
225 to conspecific odors than small males of the control group, yet no effect was detected in large
226 males. An explanation would be that small males received a higher dose with respect to their size
227 than large males. This hypothesis is unlikely because the effects of AVT treatment on behavior and

228 endurance did not vary between small and large males even though threshold-effects may exist. We
229 believe it is necessary to determine the information conveyed by body size in this species in order to
230 interpret this result. Size of individuals may reflect both age and body condition. These traits may
231 convey an integrated signal about acquisition of energetic resources of past years (Meylan et al.,
232 2002). Consequently, small males may be assumed to be either young individuals who are
233 inexperienced in terms of competition with other males or males in poor physical condition. The
234 best strategy for this type of individual (low competitive ability) may be to avoid potential agonistic
235 interactions with conspecifics in some contexts. In contrast, the absence of AVT effect on social
236 interaction in large males can be explained by the fact that for these individuals, the costs of social
237 interaction do not necessarily exceed the benefits obtained.

238 The physiological mechanism by which AVT influences social behavior is difficult to
239 identify. One possibility is that AVT affects levels of testosterone, the main steroid hormone
240 associated with dominant social behaviors, e.g., aggression and territoriality. Indeed, we observed a
241 negative effect of AVT on testosterone level but in all males, not only in small individuals. Thus, it
242 is possible that AVT acts partly through its influence on testosterone levels but it is not sufficient to
243 explain our result. Another hypothesis could be that AVT affects sensory processing. Rose and
244 Moore (2002) proposed that AVT acts on sensory pathways to modulate the responsiveness of
245 neurons to behaviorally relevant sensory stimuli and also can act on motor pathways in the
246 brainstem to modulate the neuronal output to behavior specific pattern generators. In our behavioral
247 test, because we used odor to mimic the presence of conspecific, if AVT acts on sensory pathways
248 it should be through olfaction. Young (2002) suggested that neural circuitry associated with odor
249 recognition is mediated by social neuropeptides, oxytocin and AVP. Moreover, findings of Arakawa
250 et al. (2010) provide evidence for an essential role of AVP in regulation of approach or avoidance
251 behavior in social odorant communication. Moreover, the available literature indicates that lacertids
252 are capable of finer chemosensory discrimination than was previously recognized (e.g. Léna & de
253 Fraipont 1998; Aragón, López & Martín 2001; Aragón *et al.* 2006). The site, or more likely sites,

where AVT acts to modulate sensorimotor responses to olfactory information are still unknown.

Physiological adjustments induced by AVT and stress response

Reptiles exhibit a range of physiological and behavioral adjustments to maintain homeostasis. AVT is recognized as a physiological antidiuretic hormone in reptiles (Bradshaw and Bradshaw, 2002; Bradshaw and Rice, 1981; Dantzler, 1967), but it has also been proposed that AVT functions to limit water loss through its effect on thermoregulation (Bradshaw et al., 2007). In ectothermic species, thermoregulatory behavior is the main way to regulate body temperature, but this also entails a traded-off with water conservation. In the present study, males responded to an AVT injection by increasing the time spent basking relative to control males, which contradicts previous results (e.g. Ladyman et al. 2006). The observed differences between the patterns emerging in our study and previous work may relate to differences in the habitat occupancy of species used to assess the role of AVT. The two studies that showed lowered thermoregulation included species (an agamid lizard, *Ctenophorus ornatus*, (Bradshaw et al., 2007) and elapid snake, *N. scutatus* (Ladyman et al. 2006) living in arid environments where water conservation is essential to survive. However, the typical habitat of *Z. vivipara* is a peatbog, which is a mesic environment. In this instance, a decrease in thermoregulatory behavior does not seem the suitable strategy, because maintaining body temperatures at or near physiological optimum may be more critical than water balance. Despite the cost of thermoregulation, maintaining body temperature at a narrow range of values may be selectively advantageous given that many critical physiological activities, e.g., locomotion, foraging performance and digestive assimilation, are temperature- dependent in ectothermic species (Hertz et al., 1988; Huey, 1982; Huey and Stevenson, 1979). In this context, we observed that the increase of thermoregulatory behavior by AVT injection is also associated with an enhancement of endurance. This result is in accordance with those found in bullfrog, *Rana catesbeiana* (Boyd, 1991) and female *Zootoca vivipara* (Bleu et al., 2013), but not with results found in European starling (*Sturnus vulgaris*) where AVT administration decreased heart rate and

280 was then further linked with diminished activity (Nephew et al., 2005a; Nephew et al., 2005b).

281 In addition to the different influences of AVT on the social behavior found in mammals, our study

282 demonstrates that AVT can also affect thermoregulation and activity differently depending on the

283 context. AVT seems to act to coordinate a variety of behaviors for enhancing survival. These results

284 may be related with previous results obtained in this species in studies on stress (Cote et al., 2006;

285 Cote et al., 2010; de Fraipont et al., 2000; Meylan et al., 2010), where we have demonstrated that a

286 chronic elevation of corticosterone increases energetic expenditure, food consumption, activity and

287 basking behavior. This is not so surprising since AVT has major roles in the endocrine stress

288 response by stimulating the release of adrenocorticotropin (ACTH) (Antoni, 1993; Castro et al.,

289 1986). ACTH then stimulates the release of corticosterone, the stress hormone. Finally, AVT can

290 also interact with gonadal steroids to regulate social behavior (Choleris et al., 2013). Given that

291 testosterone has been intensively associated with social behavior in males (Beletsky et al., 1989;

292 Gleason et al., 2009; O'Connell and Hofmann, 2012; Soma et al., 2008; Wingfield et al., 1990), we

293 expected that there might be differences in testosterone levels between the control and AVT-injected

294 males. Even if there is evidence that AVT exerts a direct inhibitory effect on the gonads in

295 vertebrates, its effects are not consistent between studies and biological models (Rodriguez and

296 Specker, 1991; Yamashita et al., 1980). Our results suggest that AVT has a role in regulating

297 testosterone production. We induced a decrease of testosterone level six days after AVT injection.

298 As the inhibition of aggressive behavior in young males by AVT injection, this decrease of

299 testosterone production may be a way to keep the energy for vital activities in our species. These

300 delayed effects of AVT treatment on physiology and behavior, may sound surprising but have

301 already been observed (Sangiao-Alvarellose et al. 2006) and we cannot exclude that other hormones

302 such as glucocorticoids are involved in these changes following AVT injection. By continuing to

303 examine species differences in the actions of AVT on social behavior and physiology, we will gain

304 important insights into the mechanisms that are conserved across species.

305

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479 **LIST OF FIGURE :**

480 **Figure 1. Social behavior:** Social behavior was evaluated as the response of male to the presence
481 of conspecific odors 3 days after the AVT treatment. AVT males ($n = 9$, closed circles) were injected
482 with 40 μL of a solution at 0.02 $\mu\text{g}/\mu\text{L}$ AVT diluted in sterile phosphate-buffered saline and control
483 males ($n = 11$, open triangles) with 40 μL of PBS. The response to conspecific odor was calculated
484 as the time spent on blotting paper when impregnated with odors minus the time spent on the
485 blotting paper without odor (expressed in seconds on the axis). There is a significant interaction
486 between the treatment and the snout-vent length of males (GLM procedure of SAS $p=0.04$). Small
487 males of the AVT group avoided more the conspecific odors than small control males whereas there
488 is no difference between experimental groups for larger males. The slopes are the estimates from the
489 statistical model.

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491 Figure 1

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