Insect homolog of oxytocin/vasopressin associated with parenting of males but not females in a subsocial beetle

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ABSTRACT

Parental care is thought to evolve through modification of behavioral precursors, which predicts that the mechanistic changes occur in the genes underlying those traits. The duplicated gene system of oxytocin/vasopressin has been broadly co-opted across vertebrates to influence parenting, from a pre-duplication ancestral role in water balance. It remains unclear whether co-option of these genes for parenting is limited to vertebrates. Here, we experimentally tested for associations between *inotocin* gene expression and water balance, parental acceptance of offspring, and active parenting in the subsocial beetle *Nicrophorus orbicollis*, to test whether a single copy homologue, *inotocin*, has similarly been co-opted for parental care in a species with elaborate parenting. As expected, *inotocin* was associated with water balance in both sexes. *Inotocin* expression increased around sexual maturation in both males and females, although more clearly in males. Finally, we found that expression of *inotocin* was not associated with acceptance of larvae but was associated with a transition to male but not female parenting. Moreover, level of offspring provisioning behavior and gene expression were positively correlated in males but uncorrelated in females. Our results suggest a broad co-option of this system for parenting that may have existed prior to gene duplication, and that inotocin may be associated with flexibility in parenting behavior.

52 **IMPACT SUMMARY**

53

54 Oxytocin/vasopressin are amongst the most studied neuropeptides in vertebrates, influencing
55 water balance, mating interactions, and most notably, social bonding. This gene pair evolved from
56 a duplication in the vertebrate lineage of an ancestral vasopressin-like gene. Are the multiple social
57 effects in vertebrates due to this duplication, or are social influences also ancestral? Here, we
58 demonstrate that, in a biparental social beetle with a single copy, inotocin is associated with social
59 interactions between fathers and offspring as well as being associated with the ancestral role of
60 water balance in both males and females. In vertebrates, both oxytocin and vasopressin have been
61 shown to impact social interactions in both sexes, although often showing sex-specificity in their
62 action within species. Our results suggest that this system may have been co-opted for parenting
63 prior to gene duplication and may facilitate flexibility in caring behavior.

64

Introduction

Complex behaviors like parental care evolve through the co-option of existing behaviors and their underlying genetic mechanisms (Tallamy 1984; Cunningham et al. 2017; Benowitz et al. 2018; Moore and Benowitz 2019). However, gene networks are often robust to changes in individual genes, such that multiple genetic mechanisms may produce the same phenotypes (Manceau et al. 2010; Berens et al. 2015; Barghi et al. 2019; Cunningham et al. 2021; Berendzen et al. 2022). Thus, it is unclear whether the same genes are co-opted for parental care across taxa. This is particularly the case with the neuropeptides oxytocin and vasopressin. While water balance is an ancestral function of this system (Fujino et al. 1999; Ukena et al. 2008; Koto et al. 2019), both neuropeptides have been co-opted across vertebrate taxa to influence multiple functions and are best known for their influence on parental care and especially direct parent-offspring interactions like offspring feeding (Fig. 1, SI Table 1). These effects are often sex-specific (Fig. 1; SI Table 1) and can show remarkable complementarity in their activity within and across species (Stoop 2012). Given the diversity of phenotypic effects (Fig. 1), sex-specificity, and complementarity of action of oxytocin and vasopressin, the gene duplication in vertebrates may have facilitated this diversity of gene function in social behaviors like parental care. Yet, most taxa possess only a single homologue and show similar parental care behaviors in both sexes. How ubiquitous is the co-option of these genes for complex parental care behaviors? Is the sex-specificity maintained across taxa? A test of these questions can be made using an invertebrate with parental care that has a single copy homologue of oxytocin/vasopressin.

86 A single copy homologue - inotocin – exists in insects (Aikins et al. 2008; Stafflinger et al.
87 2008). While inotocin has been broadly associated with some social contexts in insects
88 (Liutkevičiūtė et al. 2018; Muratspahić et al. 2020; Fetter-Pruneda et al. 2021; Nagy et al. 2021),
89 it is unclear whether it has been specifically co-opted to influence complex parental care behaviors
90 as most insects do not show elaborate care and prolonged parent-offspring associations as in
91 vertebrates. Burying beetles in the genus *Nicrophorus* are an exception; parents not only have
92 extended associations with their offspring abut also feed their offspring by regurgitating digested
93 carrion to begging young (Scott 1998). Both males and females can care for their offspring as
94 single parents or together. Thus, burying beetles provide a unique opportunity to determine
95 whether inotocin is associated with similar patterns of parental care as observed in vertebrates (Fig.
96 1), and an evolutionary co-option of these neuropeptides for care. Here, we focus on how *inotocin*
97 (*IT*) and *inotocin receptor* (*ITR*) expression are associated with parenting in the biparental burying
98 beetle *Nicrophorus orbicollis*.

99 All burying beetles require small animal carcasses to breed, and all exhibit extensive
100 elaborate parental care (Scott 1998). Upon finding a carcass, parents bury it, remove the exterior
101 (e.g., fur), and coat it with an exudate to prevent decomposition. Females lay eggs in the soil and
102 upon hatching, larvae crawl to the carcass. Burying beetles have temporal kin recognition; parents
103 accept and feed larvae only after a certain amount of time has elapsed since egg laying has occurred
104 (Oldekop et al. 2007). If larvae appear on the carcass too early, parents do not provide care to the
105 offspring and eat them instead. Parental care in burying beetles involves both direct (feeding of
106 regurgitated food to their offspring) and indirect parental care (defense and maintenance of
107 carcass). In *N. orbicollis*, biparental or uniparental beetles raise larvae equally effectively

108 (Benowitz and Moore 2016). Larvae of *N. orbicollis* cannot survive without parental care and
109 under biparental conditions, females are highly consistent in their parenting, while males show
110 more environmentally dependent care behavior (e.g., loss of partner; Benowitz and Moore 2016;
111 Moss and Moore 2021). Parental behavior in *N. orbicollis* thus requires egg laying, a transition
112 from infanticide to acceptance of larvae, nesting behavior, and direct larval feeding, concordant
113 with the behavioral functions of oxytocin/vasopressin in vertebrates (Fig. 1).

114 We leveraged variation across the reproductive cycle of *N. orbicollis* to address whether
115 *IT* and *ITR* gene expression in the head are associated with parenting as well as other functions of
116 the oxytocin/vasopressin in vertebrates (including water balance). As expected, *IT* and *ITR* were
117 related to water balance in both males and females. Expression also changed with sexual
118 development in both sexes. Unexpectedly, variation in *IT* expression was associated with variation
119 in male but not female parental care. Together, our results suggest that co-option of the
120 oxytocin/vasopressin system for parenting is taxonomically widespread and may have existed pre-
121 duplication.

122

123 *Material and Methods*

124

125 INSECT COLONY AND HUSBANDRY

126

127 The *Nicrophorus orbicollis* we used originated from an outbred colony regularly supplemented
128 with wild-caught beetles from Whitehall Forest, Athens, GA, USA. We maintain this colony in
129 incubators at 22 ± 1°C on a 14:10 hour light:dark cycle to simulate Georgia summer breeding

130 conditions. All individuals used were of known parentage, age, and rearing conditions. We housed
131 beetles individually at larval dispersal in 9-cm-diameter by 4-cm-deep circular compostable deli
132 containers (Eco-Products) filled with ~2.5 cm of commercial soil. Once we placed larvae into
133 individual containers, they had no further social interactions until they were collected for the age
134 series or allocated into the behavioral or physiological treatments. We defined individuals as age
135 Day 1 on the day of eclosion. We fed adult beetles organic ground beef ad libitum twice a week.
136
137 **INOTOCIN AND WATER BALANCE**
138
139 We experimentally manipulated humidity to determine whether changes in *IT* or *ITR* in the head
140 may be associated with water balance. We measured the relative humidity of 20 pairs of breeding
141 *N. orbicollis* (10 at the nest/egg stage and 10 at the larval stage) that had not been opened for one
142 week to capture the range of humidity and temperature that occurs over the breeding cycle on a
143 decomposing mouse. We used a handheld Preciva Humidity and Temperature Reader (HT154001)
144 to determine the relative humidity range. Low Humidity treatments were maintained between 8-
145 25 % (15.39 ± 1.3 % RH) and High Humidity conditions were maintained between 80-95% (84.8
146 ± 1.3 % RH). On Day 16, we transferred beetles to an individual container with perforations nested
147 inside of a breeding box (breeding boxes used throughout experiments were plastic containers;
148 Pioneer Plastics, Dixon, KY) sealed with parafilm. Individuals in the Low Humidity experiment
149 were kept without soil to ensure that they could not mitigate exposure by burrowing into the soil,
150 while individuals in the High Humidity treatment were contained in an individual container with
151 approximately two cm of potting soil. We simulated Low and High humidity conditions by

152 charging the outside box with either a) 12.5 g of $CaSO_4$ (Drierite, Hammond Drierite Co., Xena,
153 OH) for the Low Humidity treatment, or b) a 3 x 4" cotton strip soaked to saturation with deionized
154 water for the High Humidity. We left beetles in Low Humidity or High Humidity treatments for 8
155 hours to provide sufficient time for water stress to occur yet ensure that beetles survived until the
156 end of the treatment period (Bedick et al. 2006). We collected heads of n = 10 individuals of each
157 sex for each humidity treatment.
158
159 **INOTOCIN AND SOCIAL BEHAVIOR**
160
161 **Development**
162
163 We collected whole heads early post-adult eclosion (Day 2-3), pre-sexual maturity (Day 6-7), after
164 sexual maturity and early reproductive age (Day 15-16), prime reproductive age (Day 21-23), late
165 reproductive age (Day 30-31), and late-stage (Day 35-36) to test the hypothesis that *IT* changes
166 over development to enable a shift to more social life stages. This age series corresponds to the
167 timing when beetles were collected for each behavior state and the water balance experiment (SI
168 Fig. 1). We collected n = 10 individuals of each sex for all ages.
169
170 **Accept and Infanticide**
171
172 We placed a mated pair (14-15 days post-eclosion) into a plastic breeding box filled with
173 approximately two cm of potting soil moistened with distilled water and containing a freshly

174 thawed mouse (19-25 g, Rodent Pro, Inglefield, IN, U.S.A.). Breeding boxes were returned to the
175 incubator and kept on a darkened shelf, as this species breeds underground. We checked breeding
176 boxes for eggs every 24 hours, and once eggs were observed (first batch), we moved the focal
177 female and the brood ball to a new breeding box filled with approximately two cm of potting soil.
178 The focal male was kept separate from the female and brood ball for 24 hours and then we returned
179 the male to the breeding box with the female. Once the male and female were reunited, breeding
180 boxes were again checked daily for new eggs (second batch).

181 We tested the hypothesis that *IT* influences the transition from aggression to affiliation
182 towards larvae by manipulating the temporal recognition system of *Nicrophorus* (Oldekop et al.
183 2007; Benowitz et al. 2015), as parents will eat larvae that arrive before the usual embryonic period
184 is complete (~ 96 hours in *N. orbicollis*). A pair was moved to a new box following egg laying
185 (see above) to ensure that larvae did not arrive naturally at the carcass. We then introduced larvae
186 to the focal pair either too early (~72 hours after first batch of eggs laid) or at the appropriate time
187 (96 hours after egg-laying) and scored if larvae were eaten or accepted. For both treatments, we
188 provided the breeding pair with ten larvae. Following the addition of larvae, we observed the focal
189 individuals for at least 30 minutes to detect acceptance or infanticide of larvae. If the focal
190 individuals did not respond in this time, we checked pairs every 30 minutes to identify the onset
191 of acceptance or infanticide. In some cases, one parent would accept larvae while the other parent
192 committed infanticide; in these cases, we sampled and categorized the male and female separately
193 according to the phenotype that was observed for each. We collected focal individuals while
194 attacking or eating larvae for the Infanticide treatment; individuals in the Accept treatment were
195 collected immediately after the parent made antennal contact with the larvae and a feeding event

196 was observed. This manipulation often led to a discrepancy in the temporal cues used by males
197 versus females, as females base their window of larval acceptance on when eggs were laid
198 (Oldekop et al. 2007), and males likely base their window on timing and frequency of female
199 acceptance of mating. Thus, to reach an adequate sample size, we removed the female after the
200 first batch of eggs were laid, which increased the likelihood that males would accept larvae. We
201 collected n = 20 for both sexes in both treatments.
202
203 **Parental Care**
204
205 We performed four experimental treatments: Nesting (pair mated and have prepared the mouse
206 "nest", no larvae), Care (mated, have nest, and are providing care to larvae), Post Care (mated,
207 have nest, and have provided care until larvae disperse), and Double Care (mated, have nest and
208 larvae for two breeding attempts) to test the hypothesis that *IT* or *ITR* influence parental care. We
209 collected n = 20 of each treatment.
210 We collected whole heads of males and females 24-36 hours after the male had been
211 reunited with the female for the Nesting treatment. For individuals in the Care treatment, we
212 allowed eggs from the second batch to hatch and crawl to the brood ball naturally to ensure parental
213 acceptance of larvae. We checked each breeding box every 12-24 hours to determine when larvae
214 arrived. After parents interacted with their larvae for 24 hours, we scored parental care behavior
215 (see *Behavioral observations*). We collected whole heads of the focal male and female 24 hours
216 after the larvae were first observed on the carcass (at age Day 21-23). Individuals in the Post Care
217 treatment were allowed to complete parenting. Following the dispersal of larvae away from the

218 carcass and the cessation of parental care, we housed the male and female singly in individual
219 containers with soil for 24 hours prior to collection.

220 To determine whether changes of *IT* and *ITR* expression were directly related to the
221 expression of care, and/or organized the capacity for expression of parental behavior across the
222 lifetime, pairs were allowed to breed twice, creating a Double Care treatment. We removed Double
223 Care beetles for 24 hours following larval dispersal of first breeding attempt, and then put the pair
224 with a new mouse. We allowed the pair to breed normally and monitored the set up every 24 hours
225 to determine egg lay date and the arrival of larvae. 24 hours after hatch, we performed the
226 behavioral observations following the same methods as the Care treatment (see *Behavioral*
227 *Observations*). We provided a new mate to the focal individual when mortality of one parent
228 occurred during or immediately after first breeding attempt (n = 7 of 20 breeding attempts).

229

230 **Behavioral Observations**

231

232 We conducted focal behavioral observations to determine how variation in parental care was
233 related to variation in *IT* and *ITR* by observing each pair in the Care and Double Care treatments.
234 We removed lids to breeding boxes 30 minutes prior to observation to allow pairs to acclimate and
235 conducted all observations under red lights, as *N. orbicollis* are nocturnal (Wilson et al. 1984). All
236 observations were by a single observer. We recorded direct care (male or female regurgitated food
237 to larvae) and indirect care (carcass cleaning and maintenance). We also recorded time spent
238 showing "no care", defined as the parent on the carcass but not engaged in direct or indirect care
239 or was off the carcass. We collapsed these data to align with previous research using scan sampling,

240 where we recorded the activity of each beetle every minute for the 30-minute observation. This
241 gave each individual three scores (direct care, indirect care, and no care) which together summed
242 to 30. Continuous observation allowed for the observer to categorize behavior into direct or
243 indirect care by placing the behavior at each time point into its functional context. Male parental
244 care is variable and often depends on the presence and activity of the female (Walling et al. 2008;
245 Benowitz and Moore 2016). For males that did not show direct parental care during the initial
246 behavioral observation, we removed females to induce male parental care, and then collected males
247 while they were showing direct care. For males that did not show either indirect or direct parental
248 care during the behavioral observations (n = 13), we removed and collected the female to initiate
249 care behavior in the males. Males were checked every 2 – 4 hours to determine when there was a
250 transition to feeding behavior. For these males, we verified direct care of larvae prior to collection
251 by observing at least one larval feeding event.
252
253 **GENE EXPRESSION ANALYSIS FOR *INOTOCIN* AND *INOTOCIN RECEPTOR***
254
255 We decapitated the focal beetle with dissecting scissors, flash freezing the whole heads in liquid
256 nitrogen (Roy-Zokan et al. 2015), and immediately placing in storage at -80 °C until RNA
257 extraction. We homogenized heads in liquid nitrogen using a mortar and pestle and then extracted
258 RNA from each sample using Qiagen's RNAeasy Lipid Tissue Mini-Kit (cat. no. 74106) following
259 (Roy-Zokan et al. 2015), including the DNase I (Qiagen) treatment. We quantified RNA using a
260 Qubit 2.0 Fluorometer (Invitrogen Corporation, Carlsbad, CA, USA) and the RNA Broad Range
261 protocols per manufacturer's instructions. We synthesized cDNA with Quanta Biosciences qScript

262 reverse transcriptase master mix (Quanta Biosciences, Gaithersburg, MD, USA) following the
263 manufacturer's instructions from 500 ng total RNA. RNA was stored at -80 °C and cDNA was
264 stored at -20 °C.

265 We performed quantitative real-time PCR (qRT-PCR) for each of our genes of interest and
266 an endogenous control genes (*gapdh*) following methods of Cunningham et al. (2014) and using
267 transcriptome of Benowitz et al. (2017). We performed qRT-PCR with SYBR I Green Master Mix
268 and a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) following the
269 manufacturer's protocol with triple technical replicates of 10 µl reactions (n = 20 for each parental
270 context, n = 10 for each water treatment and age group, for both sexes). Primers were at a working
271 concentration of at 1.33 µmol/l. We used an annealing temperature of 60° C during the
272 amplification cycles. We established the stability of endogenous reference gene amplicons by
273 visual inspection and analysis of C_T values found that *gapdh* did not vary substantially across
274 social, water, or age contexts. The Minimum Information for Publication of Quantitative Real-
275 Time PCR Experiments (MIQE) data can be found in Appendix 1.

276

277 **STATISTICAL ANALYSES**

278

279 All statistical analyses were performed using JMP PRO (v. 16.0.0) and all figures were produced
280 in SigmaPlot (v. 14.5). Results are means ± SE. We used the $\Delta\Delta C_T$ method (Livak and Schmittgen
281 2001) to convert raw expression data to relative expression values. For all analyses, we used males
282 as the comparison group for male analyses, and females as the comparison group for female
283 analyses. We used virgin Day 21 as the comparison group for the age series. For all behavioral

284 states (Accept vs. Infanticide, Nesting, Care, and Post Care, and Care vs. Double Care), we used
285 the behavioral state that occurred earliest within the experiment as the comparison group
286 (Infanticide, Nesting, and Care, respectively). We used High Humidity as the comparison group
287 for the Low vs. High Humidity experiment. Data were visually inspected for outliers. We tested
288 for the effect of Low vs. High Humidity, Accept vs. Infanticide, and Care vs. Double Care using
289 pooled t-tests, and for the effect of age and parental care states using Analysis of Variance
290 (ANOVA). We used splines for non-parametric visualization of how expression changed with age.
291 We also assessed if there was a direct association between *IT* and *ITR* expression and
292 parental care behaviors observed in behavioral observations. We pooled data from individuals in
293 the Care and Double Care treatments as there was no difference in *IT* and *ITR* expression between
294 treatments (see Results). We analyzed the association between *IT* and *ITR* expression and care
295 behavior using linear regression for individuals that were observed to feed larvae at least once
296 during the targeted behavioral observation without removing the mate (females: n = 34, males: n
297 = 16). We analyzed the association between *IT* and *ITR* expression and indirect parental care using
298 linear regression for individuals that interacted with the brood ball or larvae in any way (females:
299 n = 34, males: n = 21). For one behavioral observation, the pair buried the carcass, and the
300 observation time was thus only 25 minutes; for this reason, all behavioral data are presented as a
301 proportion of the observation time. We excluded a subset of observations (n = 6) because male and
302 female behavior was more indicative of a response to perceived competition or disruption (i.e.,
303 frequent stridulation and both individuals off the carcass exploring breeding box).
304
305 *Results*

306

307 **INOTOCIN AND WATER BALANCE**

308

309 The relative humidity of the two water balance treatments was lower and higher than the average
310 relative humidity experienced by beetles breeding with a carcass ($F_{2,57}$ = 1032.03, P < 0.0001);
311 i.e., the Low Humidity treatments averaged 15.39 ± 1.3 % RH, breeding beetles with a carcass had
312 a RH of 72.99 ± 0.79 %, and beetles in the High Humidity treatment experienced an average
313 humidity of 84.8 ± 1.3 % RH. Males in the Low Humidity treatment had higher *IT* and *ITR*
314 expression than males in the High Humidity treatment (*IT*: $t_{1,18}$ = -2.99, P < 0.008; *ITR*: $t_{1,18}$ = -
315 2.62, P < 0.02; Fig. 2A). Female beetles in Low Humidity treatments had trend towards higher *IT*
316 ($t_{1,18}$ = -1.96, P < 0.07; Fig. 2A), while Low Humidity females expressed higher *ITR* ($t_{1,18}$ = -2.12,
317 P < 0.05; Fig. 2B), than females in the High Humidity treatment.

318

319 **INOTOCIN IN SOCIAL CONTEXTS**

320

321 Both *IT* and *ITR* changed over age for males and females. Splines indicate that both increase
322 around sexual maturity, and plateau at the higher level of expression in males and decrease again
323 at higher ages in females (Figure 2C and D). Males showed increased *IT* ($F_{5,54}$ = 3.45, P = 0.009;
324 Fig. 2C) and *ITR* ($F_{5,54}$ = 3.90, P = 0.004; Fig. 2C) expression across this timespan. In females,
325 neither the expression of *IT* ($F_{5,54}$ = 1.27, P = 0.30; Fig. 2C) nor *ITR* ($F_{5,54}$ = 1.44, P = 0.22; Fig.
326 2D) changed with adult age, likely because of the decrease at 30 and 35 days of age.

327 Variation in male parental care was associated with *IT* expression. While males that
328 committed infanticide did not differ in *IT* or *ITR* expression compared to males that accepted larvae
329 (*IT*: $t_{1,38}$ = -0.84, P = 0.41; *ITR*: $t_{1,38}$ = -0.08, P = 0.94), males in the Care treatment had higher *IT*
330 than males in the Nesting and Post Care treatments ($F_{2,57}$ = 4.23, P = 0.02, Fig. 3A; but not *ITR*:
331 $F_{2,57}$ = 0.24, P = 0.79). We found no effect of male parental state on *ITR* ($F_{2,57}$ = 0.24, P = 0.79).
332 Moreover, males that spent more time feeding larvae had higher *IT* expression than males that fed
333 less ($F_{1,14}$ = 8.91, r^2 = 0.39, P < 0.01; Fig. 3C). There was no relationship between the amount of
334 time males spent feeding larvae and *ITR* ($F_{1,14}$ = 0.08, r^2 = 0.005, P = 0.79). Amount of indirect
335 care provided was negatively correlated with *IT* expression in males ($F_{1,19}$ = 4.33, r^2 = 0.19, P =
336 0.05; Fig. 3C), but there was no relationship between *ITR* and indirect care by males ($F_{1,19}$ = 0.06,
337 r^2 = 0.003, P = 0.80). Males parenting for the first time did not have higher *IT* or *ITR* than males
338 parenting for a second time (*IT*: $t_{1,38}$ = -0.62, P = 0.54; *ITR*: $t_{1,39}$ = 0.56, P = 0.58).
339 In contrast to males, we found no significant difference in gene expression corresponding
340 to female parenting. Females that committed infanticide did not differ in *IT* or *ITR* expression
341 relative to females that accepted and cared for larvae (*IT*: $t_{1,38}$ = 1.49, P = 0.14; *ITR*: $t_{1,38}$ = 0.34, P
342 = 0.74). Female parental care states were not associated with *IT* ($F_{2,57}$ = 0.02, P = 0.98; Fig. 3B) or
343 *ITR* expression ($F_{2,57}$ = 0.21, P = 0.81). There was no relationship between the proportion of time
344 females spent feeding larvae and *IT* or *ITR* expression (*IT*: $F_{1,32}$ = 0.17, r^2 = 0.005, P = 0.69, Fig.
345 3D; *ITR*: $F_{1,32}$ = 3.15, r^2 = 0.09, P = 0.09). There was no relationship between indirect care provided
346 by females and either *IT* ($F_{1,32}$ = 0.11, r^2 = 0.003, P = 0.74, Fig. 3D) or *ITR* ($F_{1,33}$ = 0.0004, r^2 < 0.
347 0001, P = 0.98). Females parenting for the first time did not have higher *IT* or *ITR* than females
348 parenting for a second time (*IT*: $t_{1,38}$ = 0.37, P = 0.71; *ITR*: $t_{1,37}$ = -0.14, P = 0.89).

349

350 *Discussion*

351

352 Our results demonstrate that inotocin system in *N. orbicollis* is associated with both non-social and
353 social contexts important for the oxytocin/vasopressin system more broadly. As expected, we
354 found that *IT* is associated with water balance in both sexes (Fig. 2), supporting other research
355 indicating that water balance was an ancestral function of the oxytocin/vasopressin system (Fig.
356 1; Fujino et al. 1999; Ukena et al. 2008; Koto et al. 2019). We further found that *IT* is associated
357 with parenting, like other homologues in the oxytocin/vasopressin system (Fig. 1), but that the
358 associations differ between males and females.

359 We found that *IT* expression changes as beetles transition to sexual maturity. While the
360 pattern was found in both sexes, it was more pronounced in males than in females (Fig. 2). Pre-
361 reproductive males had lower *IT* expression than post-sexual maturity males, where it plateaued.
362 There was a similar increase in female *IT* expression around sexual maturity. Across vertebrate
363 species, it has been repeatedly demonstrated that oxytocin/vasopressin systems develop in a way
364 that influences subsequent expression of reproductive and parenting behaviors (e.g., Hammock
365 2015; Vaidyanathan and Hammock 2017). Based on conserved functions observed across
366 vertebrates, it is possible that *IT* is involved in priming beetles for reproduction, but further
367 research is needed to determine how the development of inotocinergic system is related to
368 reproduction.

369 Rather than a general role in parental care, we found that increased *IT* expression was
370 associated with variation in male but not female parenting in *N. orbicollis*. This holds for both

371 transition from nesting to offspring care and back to a non-parenting state, as well as overall
372 amount of time attending to offspring and performing indirect care activities such as carcass
373 maintenance (Fig. 3). Overall willingness to accept or commit infanticide of larvae was not
374 associated with differences in *IT* or *ITR* expression in either sex, suggesting that *IT* has a role
375 limited to interactions with larvae during direct care. It is possible that *IT* also has a role in female
376 parental care that is obscured because female care is less variable than the care provided by males.
377 One way to resolve this problem is to determine where *IT* is expressed in the brains of females and
378 males, as research in other systems has found that variation in sex-specificity of neuropeptides can
379 be enabled by variation in where the gene is expressed. Previous work has indicated that *IT* is often
380 produced in the SEZ, and *IT* in this location is likely to be related to water balance pathways
381 (Aikins et al. 2008; Fetter-Pruneda et al. 2021). However, it is unknown where *IT* is expressed in
382 the brains of parenting insects, and future research should determine whether *IT* is expressed in
383 similar areas of the brain in both females and males in different care states.

384 There are three potential explanations for our results: first, that a role in male parenting
385 arose de-novo in burying beetles. This is possible but not parsimonious given the distribution of
386 social influences of oxytocin/vasopressin homologues across vertebrate taxa (Fig. 1). Second,
387 given that both male and female parental behaviors are influenced by the oxytocin/vasopressin
388 system in vertebrates, it could be that the female role of *IT* was lost in burying beetles. However,
389 this is unlikely, given that other studies of female insects have not found a definitive influence of
390 *IT* on parenting behavior. Although changes in *IT* have been documented across a reproductive
391 cycle and putatively associated with parenting (Nagy et al. 2021) and other social behaviors
392 (Fetter-Pruneda et al. 2021), there has been no other experimental manipulation of parental care

393 state relative to *IT* or *ITR*. Finally, it may be that influences on social behavior are ancestral, but
394 we only see an association between male care and *IT* because *IT* is associated with flexibility in
395 social behavior. Male parental care is much more variable than female care in *N. orbicollis.* Genes
396 of the oxytocin/vasopressin family have a complex relationship with social behavior and likely
397 involve multiple genes that perform functionally redundant roles (Berendzen et al. 2022). Yet
398 another explanation is that *IT* shows similar evolutionary lability in the sex-specificity of social
399 effects as oxytocin/vasopressin do in vertebrate species (Fig. 1), although large-scale functional
400 assays across invertebrate species are required to test this.

401 There is evidence across taxa that support the hypothesis that the oxytocin/vasopressin
402 system influences flexibility in behavior. In *Caenorhabditis elegans*, the single copy homologue
403 for oxytocin/vasopressin, *nematocin,* influences male mating (Garrison et al. 2012) and gustatory
404 associative learning overall (Garrison et al. 2012; Scott et al. 2017). Oxytocin/vasopressin have
405 been found to increase the salience of social signals and group formation in a context-dependent
406 manner in both humans and fish (Kosfeld et al. 2005; Reddon et al. 2012), and are associated with
407 plasticity in socially-mediated behaviors in reef fish (e.g., Godwin et al. 2000). *IT* may perform a
408 similar role and acts to organize parental care behavior in *N. orbicollis* by increasing the relevance
409 of offspring cues to males. Support for this idea is provided by our result that males with higher
410 *IT* fed their larvae more (i.e., increased attention to larvae) than males with lower *IT*, and males
411 with lower *IT* spent more time engaged in indirect care, where attention was focused on the
412 maintenance of the carcass instead of larvae. Thus, the influence of *IT* on male parental care may
413 be more apparent because male parental provisioning is more responsive to environmental context
414 than female care (Smiseth et al. 2005; Moss and Moore 2021). If *IT* is involved in mediating

415 flexibility of parenting, this may explain why a widespread role for IT and parenting has not been
416 found, as many insects with parental care often show little flexibility in the performance of care.
417 For example, while workers transition from feeding and caring for larvae to foraging in eusocial
418 insects, they often do so in a temporal fashion moving from one task to the next in a developmental
419 series. This limits the scope for determining how variation in behavior is associated with variation
420 in *IT* expression.

421 Our results also raise questions about the role of gene duplication in behavioral evolution.
422 The vertebrate nonapeptides oxytocin and vasopressin evolved from a tandem duplication event
423 of a vasopressin-like gene (Van Kesteren et al. 1995; Goodson 2008; Theofanopoulou et al. 2021)
424 that occurred approximately 500 million years ago in the common ancestor of vertebrates
425 (Yamashita and Kitano 2013). While the role of this system in parenting has been demonstrated
426 across a wide diversity of vertebrate taxa (Fig. 1, SI Table 1), both peptides demonstrate great
427 evolutionarily lability in which function is associated with males and females (Donaldson and
428 Young 2008; Goodson and Thompson 2010; Kelly and Goodson 2014; Kohl et al. 2018). The
429 breadth of functions associated with oxytocin/vasopressin and their sex-specificity was thought to
430 be a result of gene duplication, as gene duplication is thought to facilitate the evolution of new
431 gene functions. *IT* is a single copy homologue and yet also shows a sex-specific association with
432 parenting, which may indicate that the sex-specificity of these neuropeptides, or their social
433 functions, do not require gene duplication.

434 Multiple lines of evidence support the idea that the ancestral gene was more similar to
435 *vasopressin* (Van Kesteren et al. 1995; Goodson 2008) and *IT* is more structurally similar to the
436 *vasopressin* group than *oxytocin* (Stafflinger et al. 2008). Oxytocin was first discovered in female

vertebrates in association with parturition (Dale 1909), and correspondingly, there has been a greater emphasis on the role of oxytocin in females with a lesser emphasis on vasopressin and males. For this reason, the discovery that *IT* is associated with variation in male parental care behavior, but not female parental care, was unexpected. Nevertheless, the results of our study support the idea that the social influences and potentially the sex-specificity of this system are likely ancestral.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

A.L.P. and A.J.M. conceived and designed the experiments. A.L.P. conducted experiments. A.L.P., E.C.M., P.J.M., A.T.B., and C.B.C. conducted laboratory work. A.L.P. and A.J.M analyzed the data. All authors contributed to the writing of the manuscript.

Data Availability

The data underlying this article are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.q573n5tn4).

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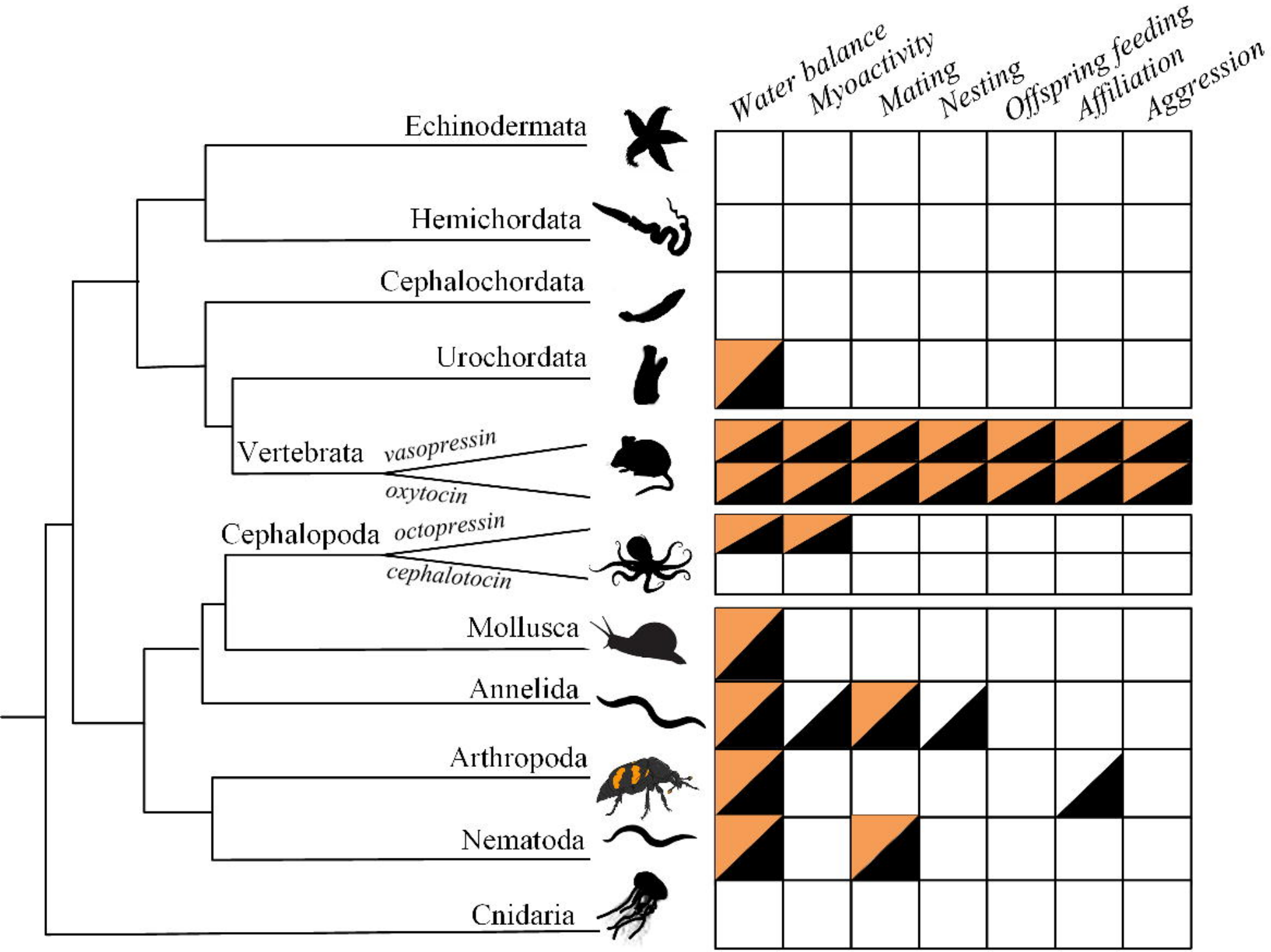
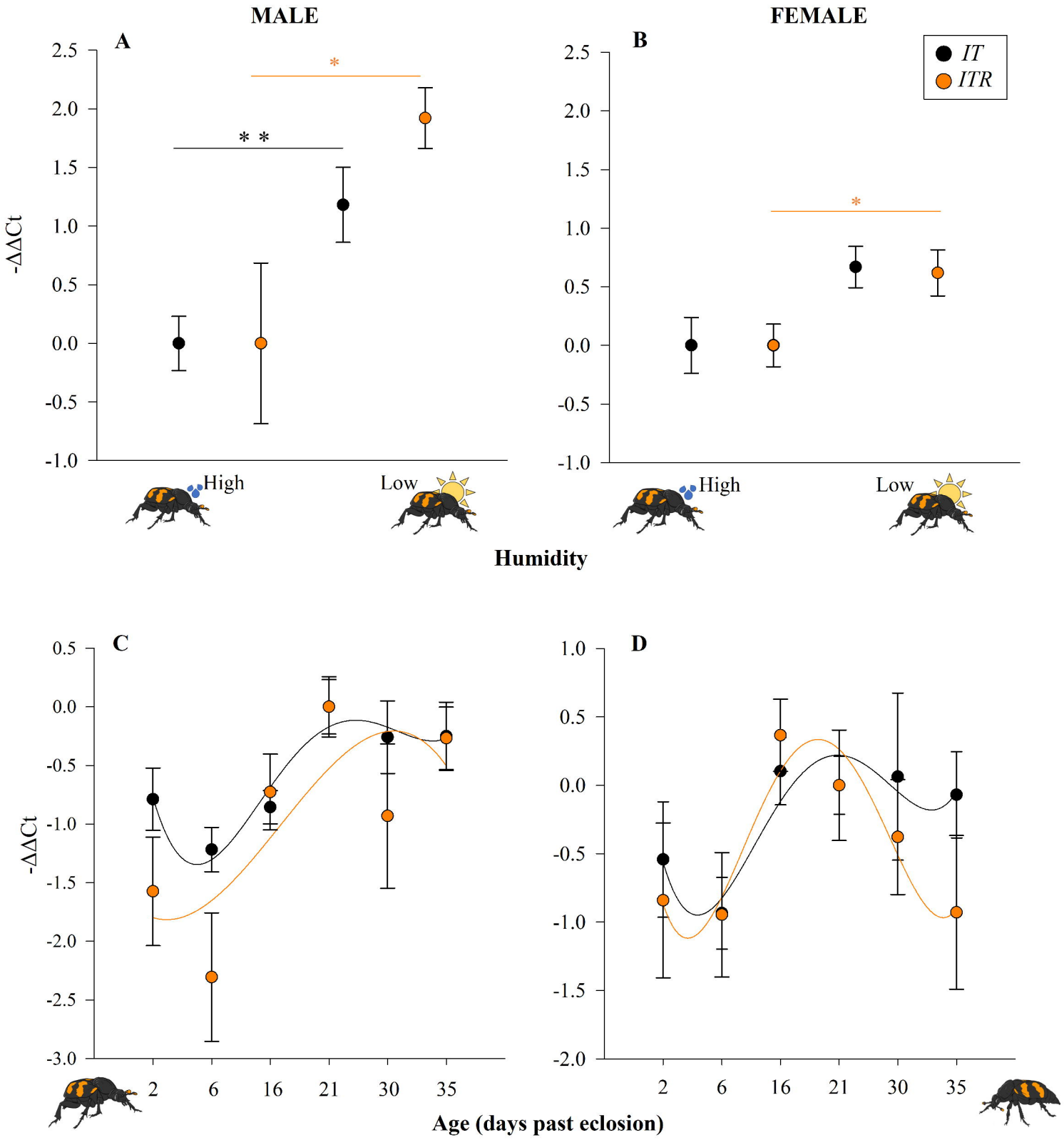
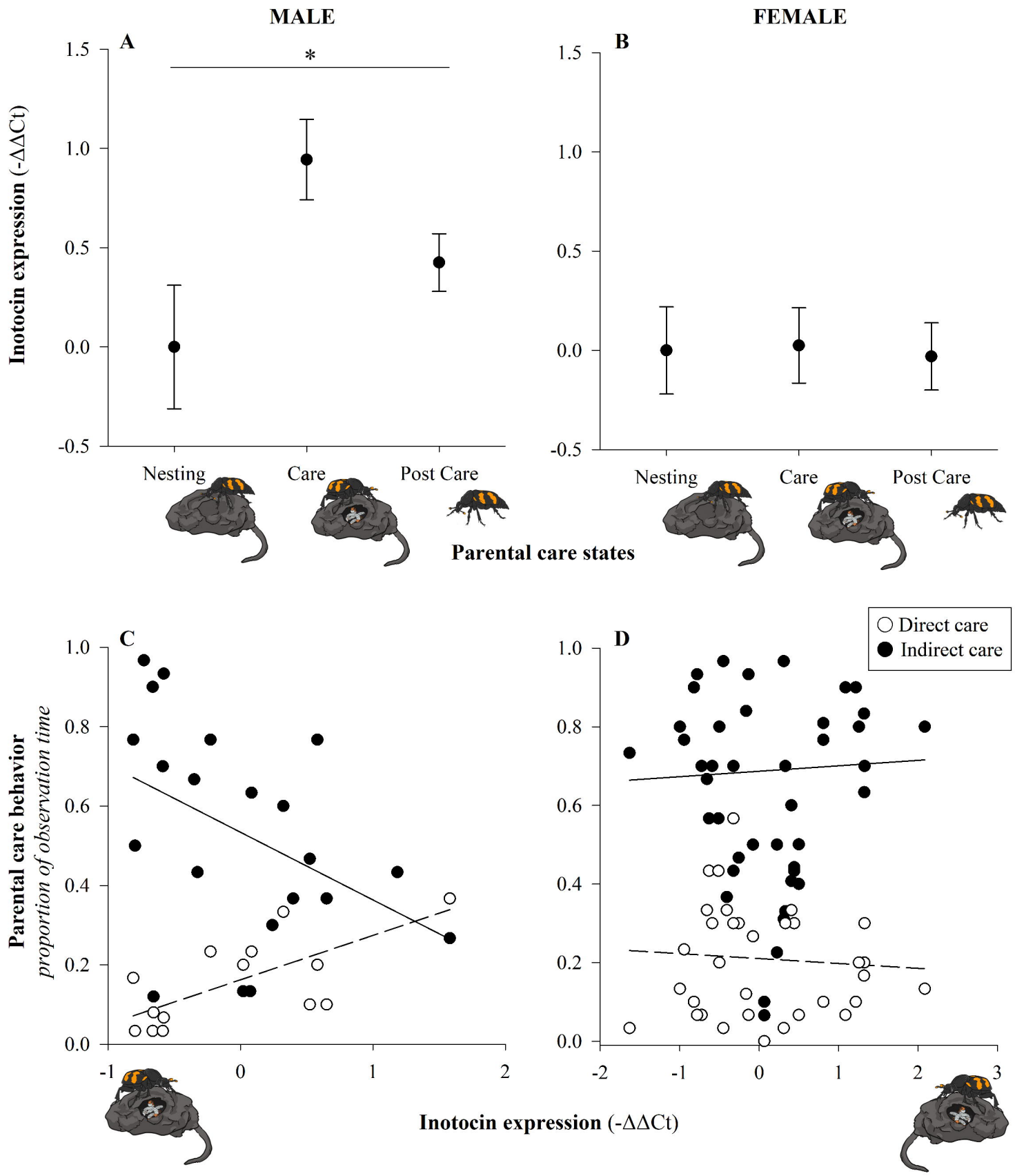
589
590 **Figure legends**
591
592 **Figure 1. Oxytocin/vasopressin family evolved from duplication event.** An ancestral
593 vasopressin-like neuropeptide was duplicated in the common ancestor of jawed vertebrates, and
594 another duplication occurred in the cephalopods. Duplication events denoted by labelled branches.
595 Neuropeptides of the oxytocin/vasopressin group have been found across wide taxonomic groups
596 (those represented on phylogeny) although the functions of these molecules are not characterized
597 for many taxa. Functions associated with these neuropeptides include water balance, myoactivity
598 (i.e., muscle activity, such as in egg laying), nesting, offspring care, affiliation, and aggression.
599 Where known, functions for these homologues are indicated next to the taxonomic group they
600 function in. Black triangles represent an association between that neuropeptide and function in
601 females, while orange triangles indicate functionality in males. Note that the branch lengths in the
602 tree are arbitrary, as well as the marked timing of the duplication events in vertebrata and
603 cephaloda. Phylogeny image modified from (Elphick et al. 2018). References supporting each
604 designation are included in Supplementary Table 1.
605
606 **Figure 2. Expression of *IT* and *ITR* genes is associated with water balance and development.**
607 Orange circles represent *IT* expression, while closed circles represent *ITR*. (***A***) *IT* and *ITR* were
608 both associated with water balance in males while (***B***) *ITR* but not *IT* was associated with water

609 balance in females. (***C***) Both *IT* and *ITR* change across the lifetime of males, corresponding to the
610 transition to sexual maturity (between Day 6 and 16). Lastly, (***D***) *IT* and *ITR* do not change across
611 the lifetime of females, although there is a trend towards a similar increase as observed in males.
612 For *A & B*, p-values are denoted over the lines, with a P-value less than 0.05 (*), and less than 0.01
613 (**). For *C & D*, spline curves were used for nonparametric visualization of these patterns. Results
614 are mean ± SEM (n = 10 for each treatment).
615
616 **Figure 3. *IT* associated with variation in male but not female parental care.** (***A***) Males showed
617 an association between parental care states and *IT* (n = 20 for each state), but (***B***) there was no
618 difference in *IT* for females across parental care states (n = 20 for each). Moreover, (***C***), for the
619 subset of males that performed care behavior without removing the female, males that fed more
620 had higher *IT* (open circles, dashed line; n = 16) and males that spent more time engaged in indirect
621 care had lower *IT* (closed circles, solid line; n = 21). Discrepancy in sample sizes reflects that
622 males that did not show any direct or indirect care were excluded from those respective analyses.
623 On the other hand, (***D***) there was no relationship between *IT* and the proportion of time females
624 spent either feeding larvae (open circles and dashed line) or engaging in indirect care (closed
625 circles and solid line; n = 34). For *A* & *B*, significant P-values are denoted over the lines, with a p-
626 value less than 0.05 denoted with a *. Results are mean ± SEM.
627


Water balance
Myoactivity
Mating
Nesting
Offspring feeding
Affiliation
Aggression
Echinodermata
Hemichordata
Cephalochordata
Urochordata
Vertebrata
vasopressin
oxytocin
Cephalopoda
octopressin
cephalotocin
Mollusca
Annelida
Arthropoda
Nematoda
Cnidaria


MALE
FEMALE
A
B
C
D
IT
ITR
-ΔΔCt
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
*
* *
High
Low
Humidity
2
6
16
21
30
35
Age (days past eclosion)


MALE
FEMALE
A
B
C
D
*
Inotocin expression (-ΔΔCt)
1.5
1.0
0.5
0.0
-0.5
Nesting
Care
Post Care
Parental care states
Direct care
Indirect care
Parental care behavior
proportion of observation time
0.8
0.6
0.4
0.2
-1
0
1
2
-2
3
Inotocin expression (-ΔΔCt)