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 behavioural 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. Yet, it is unclear whether co-option of these genes for parenting is limited to vertebrates. Here, we experimentally tested for associations between *inotocin* gene expression and parenting in the subsocial beetle *Nicrophorus orbicollis*, to test whether a species with elaborate parenting but a single copy homologue, inotocin, has similarly been co-opted for parental care. 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 associated with a transition to male but not female parenting, and that offspring provisioning behaviour and gene expression were correlated in males but not females. Using immunohistochemistry, we show that inotocin is broadly distributed in the brains of parents of both sexes. Our results suggest a broad co-option of this system for parenting that may have existed prior to gene duplication.

1. Introduction

Complex behaviors like parental care involving extensive parent-offspring interactions and provisioning of food to the offspring evolve through the co-option of existing behaviors and their underlying genetic mechanisms [1-4]. Gene networks are often robust to changes in individual genes, such that multiple genetic mechanisms may produce the same phenotypes [5-8]. Thus, it is unclear whether the same genes would be co-opted for parental care across taxa. This is particularly the case with the neuropeptides oxytocin and vasopressin, which evolved from a duplication event in the ancestor of jawed vertebrates [9-11]. While water balance is an ancestral function of this system [12-14], 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 [15]. Given the diversity of phenotypic effects (Fig. 1), sex-specificity, and complementarity of action of oxytocin and vasopressin, it seems plausible that the gene duplication in vertebrates facilitated this diversity of gene function in social behaviours like parental care. Yet, many taxa possess only a single homologue of these genes and show similar parental care behaviours 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 powerful test of these questions is to investigate whether an invertebrate with a single copy homologue of oxytocin/vasopressin shows a similar relationship between gene expression and parenting as vertebrates.

A single copy homologue of the oxytocin/vasopressin system - inotocin – exists in insects [16, 17]. While inotocin has been broadly associated with some social contexts in insects [17-19], it is unclear whether it has been specifically co-opted to influence parental care behaviors as most insects do not show elaborate care of their offspring with prolonged parent-offspring associations as observed in vertebrates. There are exceptions, notably burying beetles in the genus *Nicrophorus* where parents not only have extended associations with their offspring, they also directly provision food to their offspring by

regurgitating digested carrion [20]. Both males and females can care for their offpsring either as single parents or together. Thus, burying beetles provide a unique opportunity to determine whether inotocin is associated with similar parental care behaviours as observed in vertebrates (Fig. 1), indicting a broad evolutionary co-option of these neuropeptides for care. Here, we focus on how *inotocin* and *inotocin receptor* expression is associated with parenting in the biparental burying beetle *Nicrophorus orbicollis*.

All burying beetles are subsocial and require small animal carcasses to breed, where they perform extensive elaborate parental care [20]. Upon finding a carcass, parents bury it, remove the exterior (e.g., fur), and coat it with an exudate to prevent decomposition. Females lay eggs in the soil and upon hatching, larvae crawl to the carcass. Burying beetles identify their larvae temporally; parents will accept and feed larvae only after a certain amount of time has elapsed since egg laying has occurred [21]. If larvae appear on the carcass too early, parents do not provide care to the offspring and eat them instead. Parental care in burying beetles involves both direct (feeding of regurgitated food to their offspring) and indirect parental care (maintenance of carcass while larvae are present but without social interactions). In *N. orbicollis*, parental care can be biparental or uniparental, and females and males can both raise larvae effectively [22]. In general, when biparental, males trade-off indirect care for direct care while females are consistent in their expression of both [23, 24]. Larvae of *N. orbicollis* cannot survive without parental care and while females are highly consistent in their parenting, males show more environmentally dependent care behaviour (e.g., loss of partner; [22, 24]). Parental behaviour in *N. orbicollis* thus requires egg laying, a transition from infanticide to acceptance of larvae, nesting behaviour, and direct larval feeding, concordant with the behavioural functions of oxytocin/vasopressin in vertebrates (Fig. 1).

We leveraged this variation across the reproductive cycle of *N. orbicollis* to address whether *inotocin* and *inotocin receptor* gene expression are associated with parenting and controlled for other functions of the oxytocin/vasopressin in vertebrates (including water balance). As expected, *inotocin* and *inotocin receptor* were related to water balance in both males and females. Expression also changed with sexual development in both sexes. Unexpectedly, variation in inotocin expression was associated with variation in male but not female parental care. Together, our results suggest that co-option of the

oxytocin/vasopressin system for parenting is taxonomically widespread and may have existed pre-duplication.

2. Methods

(a) Insect Colony and Husbandry

The *Nicrophorus orbicollis* we used in this study were part of an outbred colony that we regularly supplement with wild-caught beetles from Whitehall Forest in Athens, GA, USA. We maintain this colony in an incubator (Percival, model no. I41VLC8, Perry, IA) at 22 ± 1°C on a 14:10 hour light:dark cycle to simulate summer breeding conditions. We ensured that all individuals used for this study were of known parentage, age, and rearing conditions. We housed beetles individually at larval dispersal in 9-cm-diameter by 4-cm-deep circular compostable deli containers (EP-RDP5, Eco-Products) filled with ~2.5 cm of potting soil (Performance Organics All Purpose In-Ground Soil, Miracle-Gro). Once we placed larvae into individual containers, they had no further social interactions with other burying beetles until they were collected for the age series or until they were allocated into the behavioural or physiological treatments. Individuals were defined as age Day 1 on the day of eclosion. We fed adult beetles organic ground beef twice a week.

(b) Inotocin and Water Balance

We measured the relative humidity of 20 pairs of breeding *N. orbicollis* (10 at the nest/egg stage and 10 at the larval stage) that had not been opened for one week to capture the range of humidity and temperature that occurs over the breeding cycle on a decomposing mouse. We used a handheld digital Preciva Humidity and Temperature Reader (model no. HT154001) to determine the relative humidity range.

Based on these results, we ensured that our Low and High Humidity treatments occurred at a relative humidity below and above this average relative humidity (72.99 ± 1.16 % RH). The relative humidity of the Low Humidity treatments was maintained between 8-25 % (15.39 ± 1.3 % RH) and High Humidity conditions were maintained between 80-95% (84.8 ± 1.3 % RH; see Results). On Day 16, we transferred individuals to an individual container with approximately 2 cm of potting soil with perforations nested inside of a breeding box and sealed with parafilm. We simulated Low and High humidity conditions by charging the outside box with either a) 12.5 g of $CaSO_4$ (Drierite, Hammond Drierite Co., Xena, OH) for the Low Humidity treatment, or b) a 3 x 4" cotton strip soaked to saturation with deionized water for the High Humidity [after 25]. Previous research employing a similar set-up found that *Nicrophorus marginatus* began to die around 12 hours of exposure to a similar $CaSO_4$-charged box [25]. We therefore left beetles in Low Humidity or High Humidity treatments for 8 hours to provide sufficient time for water stress to occur yet ensure that beetles survived until the end of the treatment period. Individuals in the Low Humidity experiment were kept without soil to ensure that they could not mitigate exposure by burrowing into the soil. We measured the relative humidity and temperature of each set-up at the end of the 8-hour period. We collected n = 10 individuals of each sex for each humidity treatment.

(c) Inotocin and Social Behaviour

Development

We collected whole heads early post-eclosion (Day 2-3), pre-sexual maturity (Day 6-7), after sexual maturity and during an early reproductive age (Day 15-16), at a prime reproductive age (Day 21-23), reproductive but late stage (Day 30-31), and reproductive but very late stage (Day 35-36) to test the hypothesis that inotocin changes over development to enable a shift to more social life stages. We collected post-eclosion individuals on Day 2-3 to ensure that the head was fully melanized, as individuals

are susceptible to water loss prior to hardening of the cuticle and *inotocin* is known to influence water balance functions [12, 17]. This age series corresponds to the timing when beetles were collected for each behaviour state and the water balance experiment (Supplementary Information Fig. 1). We collected n = 10 individuals of each sex for all ages.

Accept and Infanticide

We randomly mated focal virgins by placing an unrelated male and female in a small plastic container and observed to ensure that mating occurred at Day 14-15. We moved the pair to a plastic breeding box filled with approximately two cm of potting soil moistened with distilled water and containing a freshly thawed mouse between 19-25 grams (Rodent Pro, Inglefield, IN, U.S.A.) once they mated. Breeding boxes were returned to the incubator and kept on a darkened shelf. We checked breeding boxes for eggs every twenty-four hours, and once eggs were observed, we moved the focal female and the brood ball to a new breeding box filled with approximately two cm of potting soil. The focal male was kept separate from the female and brood ball for 24 hours and then we returned the male to the breeding box with the female. Once the male and female were reunited, breeding boxes were again checked daily for eggs.

We compared males that committed infanticide with those that accepted and cared for larvae arriving on the mouse resource to test the hypothesis that *inotocin* influences the transition from aggression to affiliation towards larvae. *Nicrophorus* species identify their offspring using temporal cues, such that larvae that arrive too early on the carcass are eaten and those that arrive on time are accepted by the parents and fed [21]. We took advantage of this temporal recognition to sample adults as they committed infanticide of the larvae or accepted and fed offspring. For both treatments, we provided the breeding pair with ten larvae, at least 5 of which were their offspring from the first clutch of eggs. We supplemented broods with same-age larvae from other clutches if insufficient numbers were available from the focal pair's clutch. Following the addition of larvae, we observed the focal individuals for at least thirty minutes to detect acceptance or infanticide of larvae. If the focal individuals did not respond in

this time, we checked pairs every 30 minutes to identify the onset of acceptance or infanticide. In some cases, one parent would accept larvae while the other parent committed infanticide; in these cases, we sampled and categorized the male and female separately according to the phenotype that was observed for each. We collected focal individuals while attacking or eating larvae for the Infanticide treatment, while individuals in the Accept treatment were collected immediately after the parent made antennal contact with the larvae and a feeding event was observed. This manipulation often led to a discrepancy in the temporal cues used by males versus females, as females base their window of larval acceptance on when eggs were laid [21] and males likely base their window on timing and frequency of female acceptance of mating (unpub. data). Thus, to reach an adequate sample size, for most males in the treatment, we removed the female after the first the first batch of eggs were laid, which increased the likelihood that males would accept larvae. We collected n = 20 for both sexes in the Accept and Infanticide treatments.

Parental Care

We performed four experimental treatments including Nesting (pair mated and have prepared the mouse "nest", no larvae), Care (pair mated, have nest, and are providing care to larvae), Post Care (pair mated, have nest, and have provided care until larvae disperse), and Double Care (pair mated, have nest and larvae for two breeding attempts) to test the hypothesis that *inotocin* or *inotocin receptor* influence parental care expressed towards larvae. For all Nesting, Care, Post Care, and Double Care treatments, we followed the same initial breeding protocol as the Accept and Infanticide treatments but performed different manipulations after reintroducing the male. We collected n = 20 of each treatment for males and females.

We collected whole heads of males and females 24-36 hours after the male had been reunited with the female for the Nesting treatment. For individuals in the Care treatment, we allowed eggs from the second to hatch and crawl to the brood ball naturally to ensure parental acceptance of larvae. We checked each breeding box every 12-24 hours to determine when larvae arrived on the brood ball. After

parents interacted with their larvae for 24 hours, we scored parental care behaviour in a behavioural observation (see *Behavioural observations*). We collected whole heads of the focal male and female 24 hours after the larvae were first observed on the carcass (at age Day 21-23), while the focal individual was located on the brood ball and engaged in direct care behaviour. Male parental care is variable and often depends on the presence and activity of the female [22, 26]. For males that did not show direct parental care during the initial behavioural observation, we removed females to induce male parental care, and then collected males 24 hours later while they were showing direct care to larva. Individuals in the Post Care treatment were allowed to complete a breeding cycle. Following the dispersal of larvae away from the carcass and the cessation of parental care behaviour, we housed the male and female singly in individual containers with soil for 24 hours, at which point whole heads were collected on approximately Day 30.

To determine whether changes of *inotocin* and *inotocin receptor* expression were directly related to the expression of care, and/or organized the capacity for expression of parental behaviour across the lifetime, pairs were allowed to breed twice, creating a Double Care treatment. Double Care beetles were removed for 24 hours following larval dispersal, and then put with a new mouse under the same conditions. For the second breeding attempt, we allowed the pair to breed normally and monitored the set up every 24 hours to determine egg lay date and the arrival of larvae. Twenty-four hours after hatch, we observed the pair for 30-minutes and whole heads were collected as they were engaged in direct care behaviour (approximately Day 35). Similar to the observations performed for Care treatments, if a male did not show direct care behaviour during the initial behavioural observation, we removed the female and collected males 24 hours later while they were engaged in direct care behaviour. We provided a new mate to the focal individual when mortality of one parent occurred during or immediately after first breeding attempt (n = 7 of 20 breeding attempts).

Behavioural Observations of Parental Care

We conducted focal behavioural observations for each pair in the Care and Double Care treatments to quantify parental care. Before an observation was initiated, we removed lids to breeding boxes and the pair was given at least 30 minutes to acclimate. We conducted all observations under red lights to ensure no light effects on individual care behaviour, as *N. orbicollis* are nocturnal [27]. In each behavioural observation, we recorded both direct and indirect care [26]. Direct care is defined as when the male or female fed larvae. Indirect care is defined as activities related to carcass cleaning and maintenance, which allows for the preservation of the carcass for future offspring provisioning and thus represents a type of parental care without direct interactions with offspring. We also recorded time spent showing "no care", defined as when the parent was on the carcass but not engaged in direct or indirect care or was off the carcass. We recorded behaviours as a frequency (e.g., number of feedings) and a duration (length of time engaged in the behaviour). We collapsed these data to align with previous research using scan sampling, where the activity of each beetle every minute was recorded. This gave each individual three scores (direct care, indirect care, and no care) which together summed to 30. We used this method because specific behaviours are often difficult to disentangle, e.g., larval feeding is often interrupted by the adults "self-feeding", which is then followed by direct provisioning of larvae. For this reason, continuous observation allowed for the observer to categorize behaviour into direct or indirect care by placing the behaviour at each time point into its functional context. For males that did not show either indirect or direct parental care during the behavioural observations (n = 13), we removed and collected the female to initiate care behaviour in the males. For these males, we verified direct care of larvae prior to collection by observing at least one larval feeding event.

(d) Gene Expression Analysis for *Inotocin* and *Inotocin Receptor*

We collected samples with dissecting scissors, flash freezing them in liquid nitrogen [28], and immediately placed in storage at -80 °C until RNA extraction. A small subset of heads (n = 5), heads were collected, bisected with scissors to expose brain tissue, placed into 500 ml RNAlater (Ambion, Grand

Island, NY, USA), and stored at -20°C until RNA extraction. We homogenized whole heads in liquid nitrogen using a mortar and pestle (cat. no. 60310, CoorsTek, Golden, CO, USA). Following homogenization, we extracted RNA from each sample using Qiagen's RNAeasy Lipid Tissue Mini-Kit (cat. no. 74106) following [28], including the DNase I (Qiagen) treatment. RNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen Corporation, Carlsbad, CA, USA) using the RNA Broad Range protocols per manufacturer's instructions. We synthesized cDNA with Quanta Biosciences qScript reverse transcriptase master mix (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's instructions from 500 ng total RNA. RNA was stored at -80 °C and cDNA was stored at -20 °C.

We performed quantitative real-time PCR (qRT-PCR) for each of our genes of interest and several endogenous control genes (*gapdh* and *tbp*) following methods of Cunningham et al. [29] and using transcriptome of Benowitz et al. [30]. We performed qRT-PCR with Roche LightCycler 480 SYBR I Green Master Mix using a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's protocol with triple technical replicates of 10 µl reactions (n = 20 for each parental experimental context, n = 10 for in each water treatment, and n = 10 for each age group, for both males and females). Primers were at a working concentration of at 1.33 µmol/l. We used an annealing temperature of 60° C during the amplification cycles. We established the stability of the endogenous reference gene by running replicates from each treatment and assessing the stability of endogenous reference gene amplicons by visual inspection and analysis of C_T values found that *gapdh* did not vary substantially across social, water, or age contexts. Additional information suggested by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines can be found in Appendix 1.

(e) Immunohistochemistry of Inotocin in the Brain

We dissected brains from female and male beetles at the period of peak parental care behaviour, as described above, for immunofluorescence microscopy in ice-cold 1x PBS. We fixed brains immediately in 4% paraformaldehyde in PBT (PBS with 0.5% Triton X-100) for 24 hours at 4° C. Following fixation, we permeabilized the brains by first incubating in 2N HCl for 30 minutes at 4° C. We then rinsed brains 3 times in PBT to remove the HCl and permeabilization was continued by treating the brains in PBS with 4% Triton X-100 for 1 hour at room temperature, after which we washed brains the brains 3 times for 10 minutes in PBT. We blocked non-specific binding sites by incubating the brains in 1% bovine serum albumin in PBT overnight at 4° C.

We incubated brains in a 1:300 dilution of primary polyclonal antibody that recognizes the pre-pro-peptide of inotocin [31] for 48 hours at 4° C. For all treatments (male and female parental care), a subset of brains was not treated with primary antibody to validate the specificity of inotocin staining. We then washed the brains 5 times in PBT for 1 to 3 hours per wash step at room temperature and added secondary antibody solution (Alexa Fluor 647 donkey anti-goat, 1:500). We added 0.5 mg/mL DAPI at a 1:2000 dilution to the secondary antibody solution to counterstain for DNA and incubated the brains in secondary antibody overnight at 4° C. We washed the brains 5 times for 1 to 3 hours each step. After washing, we infiltrated the brains in mounting medium (90% glycerol in 0.1 M Tris HCl, pH 8.7) by taking the brains through a dilution series of 3:1, 1:1, 1:3 PBT: mounting medium, followed by 90% mounting medium with incubation steps of at least two hours each. We then mounted brains on depression well slides and imaged brains with a Zeiss LSM 710 Confocal Microscope at the UGA Biomedical Microscopy Core. Brains processed in the absence of primary antibody showed no background fluorescence due to non-specific binding of the secondary antibody (SI Fig. 2).

(f) Statistical Analyses

All statistical analyses were performed using JMP PRO (v. 16.0.0, http://jmp.com) and all figures were produced in SigmaPlot (v. 14.5, http://www.sigmaplot.co.uk). Results are means ± SE. We used the $\Delta\Delta C_T$ method [32] to convert raw expression data to relative expression values. For all analyses, we used males as the comparison group for male analyses, and females as the comparison group for female analyses. We used virgin Day 21 as the comparison group for the age series. For all behavioural states (Accept vs. Infanticide, Nesting, Care, and Post Care, and Care vs. Double Care), we used the behavioural state that occurred earliest within the experiment as the comparison group (Infanticide, Nesting, and Care, respectively). We used High Humidity as the comparison group for the Low vs. High Humidity experiment. Data were visually inspected for outliers. We tested for the effect of Low vs. High Humidity, Accept vs. Infanticide, and Care vs. Double Care using pooled t-tests, and for the effect of age and parental care states using Analysis of Variance (ANOVA). We used splines for non-parametric visualization of how expression changed with age.

We also assessed if there was a direct association between *inotocin* and *inotocin receptor* expression and parental care behaviours observed in behavioural observations. We pooled data from individuals in the Care and Double Care treatments as there was no difference in *inotocin* and *inotocin receptor* expression between treatments (see Results). We analyzed the association between *inotocin* and *inotocin receptor* expression and care behaviour (proportion of observation points spent in direct care) using linear regression for individuals that were observed to feed larvae at least once during the targeted behavioural observation without removing the mate (females: n = 34, males: n = 16). We analyzed the association between *inotocin* and *inotocin receptor* expression and indirect parental care using linear regression for individuals that interacted with the brood ball or larvae in any way (females: n = 34, males: n = 21). For one behavioural observation, the pair buried the carcass, and the observation time was thus only 25 minutes; for this reason, all behavioural data are presented as a proportion of the observation time. We excluded a subset of observations (n = 6) because male and female behaviour was more indicative of a response to perceived competition or disruption (i.e., frequent stridulation and both individuals off the carcass exploring breeding box).

3. Results

(a) Inotocin and Water Balance.

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

(b) Inotocin in Social Contexts.

Both *inotocin* and *inotocin receptor* changed over different ages for males and females. Using a non-parametric visualization, we can see that both increase around sexual maturity, and plateau at the higher level of expression in males and decrease again at higher ages in females (Figure 2C and D). Males showed increased *inotocin* ($F_{5,54}$ = 3.45, P = 0.009; Fig. 2C) and *inotocin receptor* ($F_{5,54}$ = 3.90, P = 0.004; Fig. 2C) expression across this timespan. In females, neither the expression of *inotocin* ($F_{5,54}$ = 1.27, P = 0.30; Fig. 2C) nor *inotocin receptor* ($F_{5,54}$ = 1.44, P = 0.22; Fig. 2D) changed with adult age, likely because of the decrease at 30 and 35 days of age.

Variation in male parental care was associated with *inotocin* expression. While males that committed infanticide did not differ in *inotocin* or *inotocin receptor* expression compared to males that accepted larvae (*inotocin*: $t_{1,38}$ = -0.84, P = 0.41; *inotocin receptor*: $t_{1,38}$ = -0.08, P = 0.94), males in the Care treatment had higher *inotocin* than males in the Nesting and Post Care treatments ($F_{2,57}$ = 4.23, P = 0.02, Fig. 3A; but not *inotocin receptor*: $F_{2,57}$ = 0.24, P = 0.79). Moreover, males that spent more time feeding larvae had higher *inotocin* expression than males that fed less ($F_{1,14}$ = 8.91, r^2 = 0.39, P < 0.01; Fig. 3C). There was no relationship between the amount of time males spent feeding larvae and *inotocin receptor* ($F_{1,14}$ = 0.08, r^2 = 0.005, P = 0.79). Amount of indirect care provided was negatively correlated with *inotocin* expression in males ($F_{1,19}$ = 4.33, r^2 = 0.19, P = 0.05; Fig. 3C), but again there was no relationship between *inotocin receptor* and indirect care by males ($F_{1,19}$ = 0.06, r^2 = 0.003, P = 0.80). We found no effect of male parental state on *inotocin receptor* ($F_{2,57}$ = 0.24, P = 0.79). Males parenting for the first time did not have higher *inotocin* or *inotocin receptor* than males parenting for a second time (*inotocin*: $t_{1,38}$ = -0.62, P = 0.54; *inotocin receptor*: $t_{1,39}$ = 0.56, P = 0.58).

In contrast to males, we found no pattern of gene expression and female parenting. Females that committed infanticide did not differ in *inotocin* or *inotocin receptor* expression relative to females that accepted and cared for larvae (*inotocin*: $t_{1,38}$ = 1.49, P = 0.14; *inotocin receptor*: $t_{1,38}$ = 0.34, P = 0.74). Female parental care states were not associated with *inotocin* ($F_{2,57}$ = 0.02, P = 0.98; Fig. 3B) or *inotocin receptor* expression ($F_{2,57}$ = 0.21, P = 0.81). There was no relationship between the proportion of time females spent feeding larvae and *inotocin* or *inotocin receptor* expression (*inotocin*: $F_{1,32}$ = 0.17, r^2 = 0.005, P = 0.69, Fig. 3D; *inotocin receptor*: $F_{1,32}$ = 3.15, r^2 = 0.09, P = 0.09). There was no relationship between indirect care provided by females and either *inotocin* ($F_{1,32}$ = 0.11, r^2 = 0.003, P = 0.74, Fig. 3D) or *inotocin receptor* ($F_{1,33}$ = 0.0004, r^2 < 0. 0001, P = 0.98). Females parenting for the first time did not have higher *inotocin* or *inotocin receptor* than females parenting for a second time (*inotocin*: $t_{1,38}$ = 0.37, P = 0.71; *inotocin receptor*: $t_{1,37}$ = -0.14, P = 0.89).

We found that parents of both sexes showed diffuse expression of inotocin in the brain (Fig. 4). Staining was concentrated in the suboesophageal zone and adjacent to the antennal lobes, in the vicinity

of the mushroom bodies. There were no obvious differences in either the pattern or density of staining in male versus female brains across individuals.

4. Discussion

In this study we leveraged inotocin, an insect homologue of the oxytocin/vasopressin system of vertebrates, to determine whether this system has been broadly co-opted to influence parenting. Our results demonstrate that inotocin in *N. orbicollis* is associated with both non-social and social contexts important for the oxytocin/vasopressin system more broadly. As expected, we found that *inotocin* is associated with water balance in both sexes (Fig. 2), supporting other research indicating that water balance was an ancestral function of the oxytocin/vasopressin system (Fig. 1; [12, 13, 33]). We further found that inotocin is associated with parenting, but that the associations differ between males and females.

We found that *inotocin* expression changes as beetles transition to sexual maturity. While the pattern was found in both sexes, it was more pronounced in males than in females (Fig. 2). Pre-reproductive males had lower inotocin expression than post-sexual maturity males, where it plateaued. There was a similar increase in female inotocin expression around sexual maturity. Across vertebrate species, it has been repeatedly demonstrated that oxytocin/vasopressin systems develop in a way that influences subsequent expression of reproductive and parenting behaviours [e.g., 34, 35]. Based on conserved functions observed across vertebrates, it is possible that *inotocin* is involved in priming beetles for reproduction, but further research would be needed to determine how the development of inotocinergic system may be related to reproduction.

Given the complex parent-offspring social interactions observed in this species, and that both sexes can raise offspring solo [22], we predicted an association between inotocin and parenting. However, rather than a general role in parental care, we found that increased *inotocin* expression was associated

with variation in male but not female parenting in *N. orbicollis*. This holds for both transition from nesting to offspring care and back to a non-parenting state, as well as overall amount of time attending to offspring and performing indirect care activities such as carcass maintenance (Fig. 3). However, these associations of *inotocin* with parenting did not hold for females; *inotocin* expression did not vary across parental care states of females, nor was variation in observed behaviour associated with variation in female inotocin. Overall willingness to accept or commit infanticide of larvae was not associated with differences in *inotocin* or *inotocin receptor* expression in either sex, suggesting that inotocin has a role limited to interactions with larvae during direct care. Despite this, immunohistochemistry revealed that brains of parenting males and females showed similar patterns of expression, with most staining occurring in the subesophageal zone (SEZ) and the vicinity of the mushroom bodies (MB; Fig. 4). Immunohistochemistry shows diffuse staining for inotocin expression across the brains of both males and female parents (Fig. 4), which supports a role for inotocin in both sexes. Previous work has indicated that inotocin is often produced in the SEZ, and inotocin in this location is likely to be related to water balance pathways [17, 31]. Mushroom bodies have been associated with social behaviour in insects more broadly [36, 37], and for this reason, the expression of inotocin in the region of the mushroom bodies may indicate that this is one mechanism by which parenting is influenced. However, future research would be needed to determine how the distribution of inotocin influences parenting in males and females.

Our data raise questions about the evolution of social influences in the oxytocin/vasopressin system. There are three potential explanations for our results: first, that a role in male parenting arose de-novo in burying beetles. This is possible but not parsimonious given the distribution of social influences of oxytocin/vasopressin homologues across vertebrate taxa (Fig. 1). Second, given that both male and female parental behaviours are influenced by the oxytocin/vasopressin system in vertebrates, it could be that the female role of inotocin was lost in burying beetles. However, this is unlikely, given that, thus far, other studies of female insects have not found a definitive influence of inotocin on parenting behaviour [31, 38]. Alternatively, it may be that influences on social behaviour are ancestral, but we only see an association between male care and inotocin because inotocin is associated with flexibility in social

behaviour. Male parental care is much more variable than female care in *N. orbicollis*. Yet another explanation is that inotocin shows similar evolutionary lability in the sex-specificity of social effects as oxytocin/vasopressin do in vertebrate species (Fig. 1), although large-scale functional assays across invertebrate species would be required to test this.

There is evidence across taxa that support the hypothesis that the oxytocin/vasopressin system influences flexibility in behaviour. In *Caenorhabditis elegans*, the single copy homologue for oxytocin/vasopressin, *nematocin*, influences male mating [38] and gustatory associative learning overall [39, 40]. Oxytocin/vasopressin have been found to increase the salience of social signals and group formation in a context-dependent manner in both humans and fish [41, 42], and are associated with plasticity in socially-mediated behaviours in reef fish [e.g., 43]. It is plausible that *inotocin* may perform a similar role and acts to organize parental care behaviour in *N. orbicollis* by increasing the relevance of offspring cues to males. Support for this idea is provided by our result that males with higher *inotocin* fed their larvae more (i.e., increased attention to larvae) than males with lower *inotocin*, and males with lower *inotocin* spent more time engaged in indirect care, where attention was focused on the maintenance of the carcass instead of larvae. Thus, the influence of inotocin on male parental care may be more apparent because male parental provisioning is more responsive to environmental context than female care [23, 24]. If inotocin is involved in mediating flexibility of parenting, this may explain why a widespread role for inotocin and parenting has not been found, as many insects with parental care often show little plasticity in the performance of care. For example, while workers transition from feeding and caring for larvae to foraging in eusocial insects, they often do so in a temporal fashion moving from one task to the next in a developmental series. This limits the scope for determining how variation in behaviour is associated with variation in inotocin expression. Determining whether homologues are broadly associated with the context-dependency of social behaviour across species like oxytocin/vasopressin in vertebrates would be a fruitful area for future research.

Our results further raise questions about the role of gene duplication in behavioral evolution. The nonapeptides oxytocin and vasopressin evolved from a tandem duplication event of a vasopressin-like

gene [9-11] that occurred approximately 500 million years ago in the common ancestor of vertebrates [44], although the oxytocin and vasopressin group of homologues are part of an ancient family of conserved peptides that have existed for over 700 million years [45, 46]. While the role of this system in parenting has been demonstrated across a wide diversity of vertebrate taxa (Fig. 1, SI Table 1), both peptides demonstrate great evolutionarily lability in which function is associated with males and females [45, 47-49]. The breadth of functions associated with oxytocin/vasopressin and their sex-specificity was thought to be a result of gene duplication, as gene duplication is broadly thought to facilitate the evolution of new gene functions. Inotocin is a single copy homologue and yet also shows a sex-specific association with parenting, which may indicate that the sex-specificity of these neuropeptides, or their social functions, do not require gene duplication. However, it is unclear how gene duplication may influence behavioral evolution because ancestral behavior often cannot be inferred from a fossil record. Determining the social and parenting roles of single copy homologues across taxa may provide inference into how gene duplication influences the evolution of behavior more broadly.

Multiple lines of evidence support the idea that the ancestral gene was more similar to *vasopressin* [9, 10] and *inotocin* is more structurally similar to the *vasopressin* group than *oxytocin*, despite its name [16]. Oxytocin was first discovered in female vertebrates in association with parturition [50], 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 inotocin is associated with variation in male parental care behaviour, but not female parental care, was unexpected. Nevertheless, the results of this study support the idea that the social influences and potentially the sex-specificty of this system are likely ancestral. Moreover, these data suggest that the role of specific genes in parenting can be predicted from their action in other taxa.

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Figure legends

Figure 1. Oxytocin/vasopressin family evolved from duplication event. An ancestral vasopressin-like neuropeptide was duplicated in the common ancestor of jawed vertebrates, and another duplication occurred in the cephalopods. Duplication events denoted by labelled branches. Neuropeptides of the oxytocin/vasopressin group have been found across wide taxonomic groups (those represented on phylogeny) although the functions of these molecules are not characterized for many taxa. Functions associated with these neuropeptides include water balance, myoactivity (i.e., muscle activity, such as in egg laying), nesting, offspring care, affiliation, and aggression. Where known, functions for these

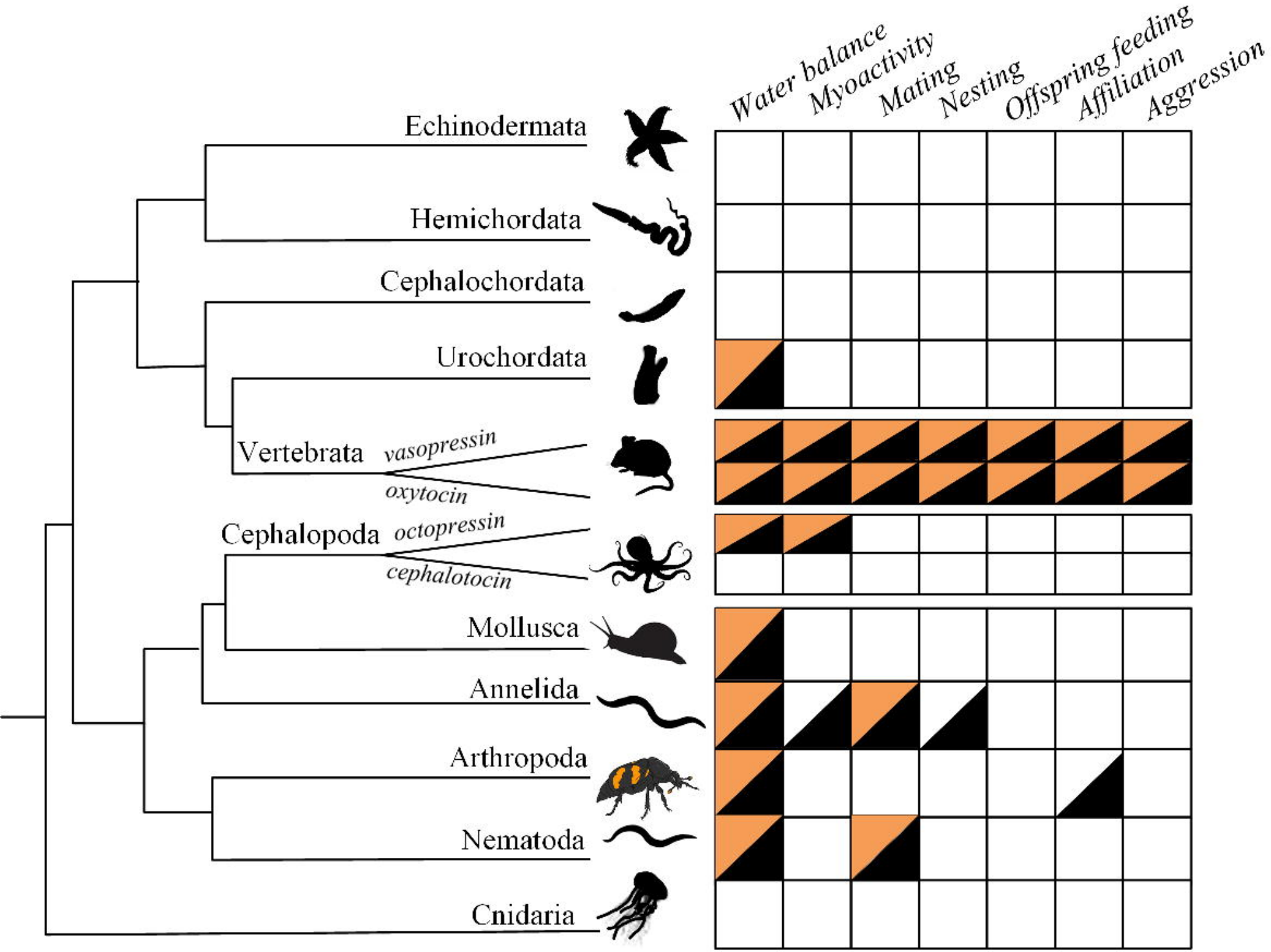
homologues are indicated next to the taxonomic group they function in. Black triangles represent an association between that neuropeptide and function in females, while orange triangles indicate functionality in males. Note that the branch lengths in the tree are arbitrary, as well as the marked timing of the duplication events in vertebrata and cephaloda. Figure modified from [51]. References are included in Supplementary Table 1.

Figure 2. Expression of *inotocin* and *inotocin receptor* genes is associated with water balance and development. Orange circles represent *inotocin* expression, while closed circles represent *inotocin receptor*. (***A***) *inotocin* and *inotocin receptor* were both associated with water balance in males while (***B***) *inotocin receptor* but not *inotocin* was associated with water balance in females. (***C***) Both *inotocin* and *inotocin receptor* change across the lifetime of males, corresponding to the transition to sexual maturity (between Day 6 and 16). Lastly, (***D***) *inotocin* and *inotocin receptor* do not change across the lifetime of females, although there is a trend towards a similar increase as observed in males. For *A & B*, p-values are denoted over the lines, with a P-value less than 0.05 (*), and less than 0.01 (**). For *C & B*, spline curves were used for nonparametric visualization of these patterns. Results are mean ± SEM (n = 10 for each treatment).

Figure 3. Inotocin associated with variation in male but not female parental care. (***A***) Males showed an association between parental care states and *inotocin* (n = 20 for each state), but (***B***) there was no difference in *inotocin* for females across parental care states (n = 20 for each). Moreover, (***C***), for the subset of males that performed care behaviour without removing the female, males that fed more had higher *inotocin* (open circles, dashed line; n = 16) and males that spent more time engaged in indirect care had lower *inotocin* (closed circles, solid line; n = 21). Discrepancy in sample sizes reflects that males that did not show any direct or indirect care were excluded from those respective analyses. On the other hand, (***D***) there a relationship between *inotocin* and the proportion of time females spent either feeding larvae (open circles and dashed line) or engaging in indirect care (closed circles and solid line; n = 34). For *A* &

C, significant P-values are denoted over the lines, with a p-value less than 0.05 denoted with a *. Results are mean ± SEM.

Figure 4. Inotocin shows diffused expression in the brains of both sexes. (***A***) The whole brain of *Nicrophorus orbicollis* stained with DAPI (blue), with the antennal lobes (AL) and suboesophageal zone (SEZ) indicated. Inotocin staining was not found in the optic lobes, and for this reason, the blue rectangle indicates the regions included in *B-D*. All images are of the frontal side of the brain and are at the same magnification as *A* unless otherwise noted. Diffuse inotocin staining (purple) was observed in the SEZ and adjacent to the ALs, in both females (***B***) and males (***C***). (***D***) A cluster of inotocin-staining cells were frequently observed in the lower SEZ in both sexes. (**E**) Inotocin staining is apparent in the dorsal view of the brain, corresponding to the general location of the mushroom bodies (MB). Direction indicated as anterior (A) to posterior (P).


Water balance
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Affiliation
Aggression
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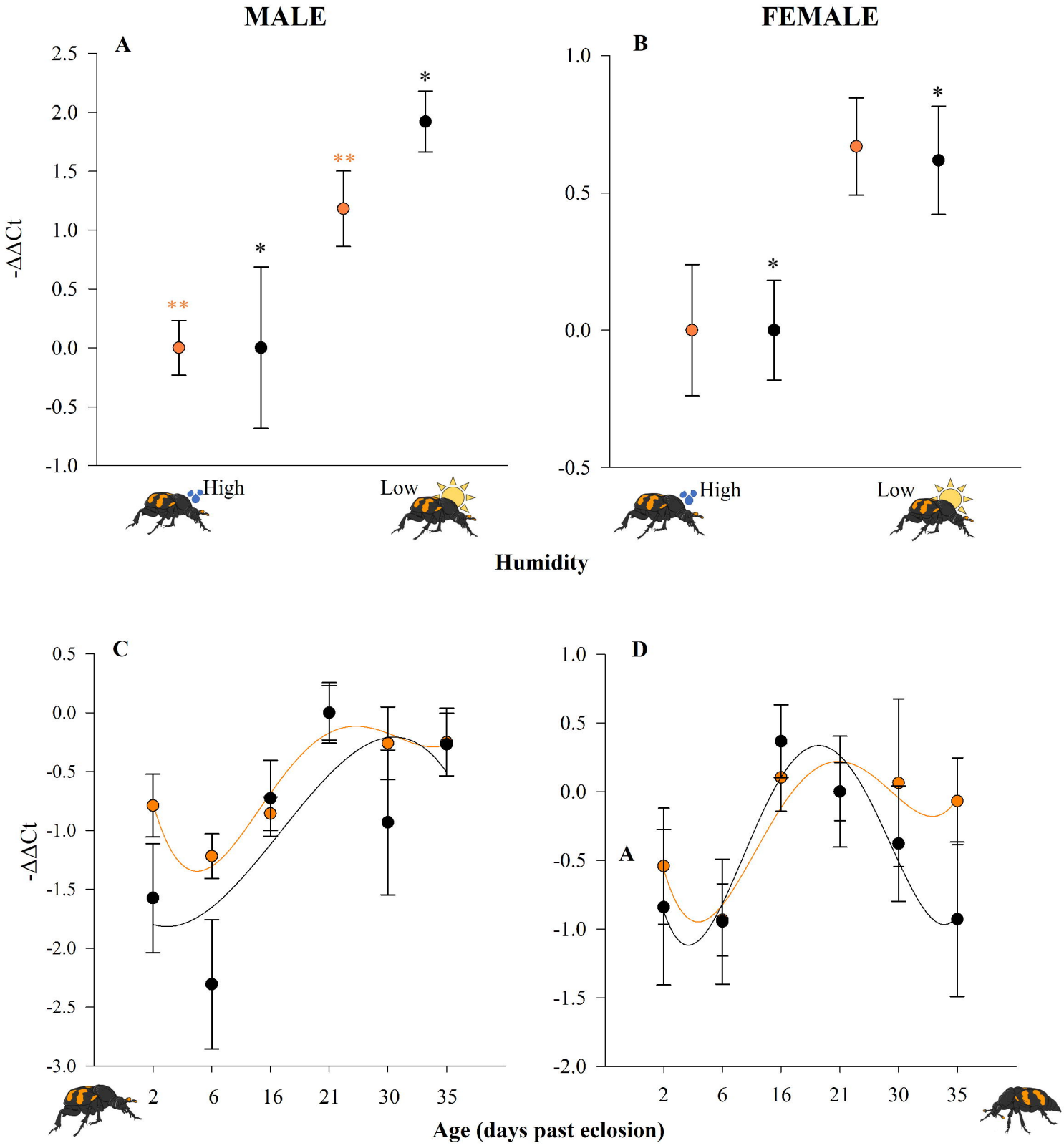
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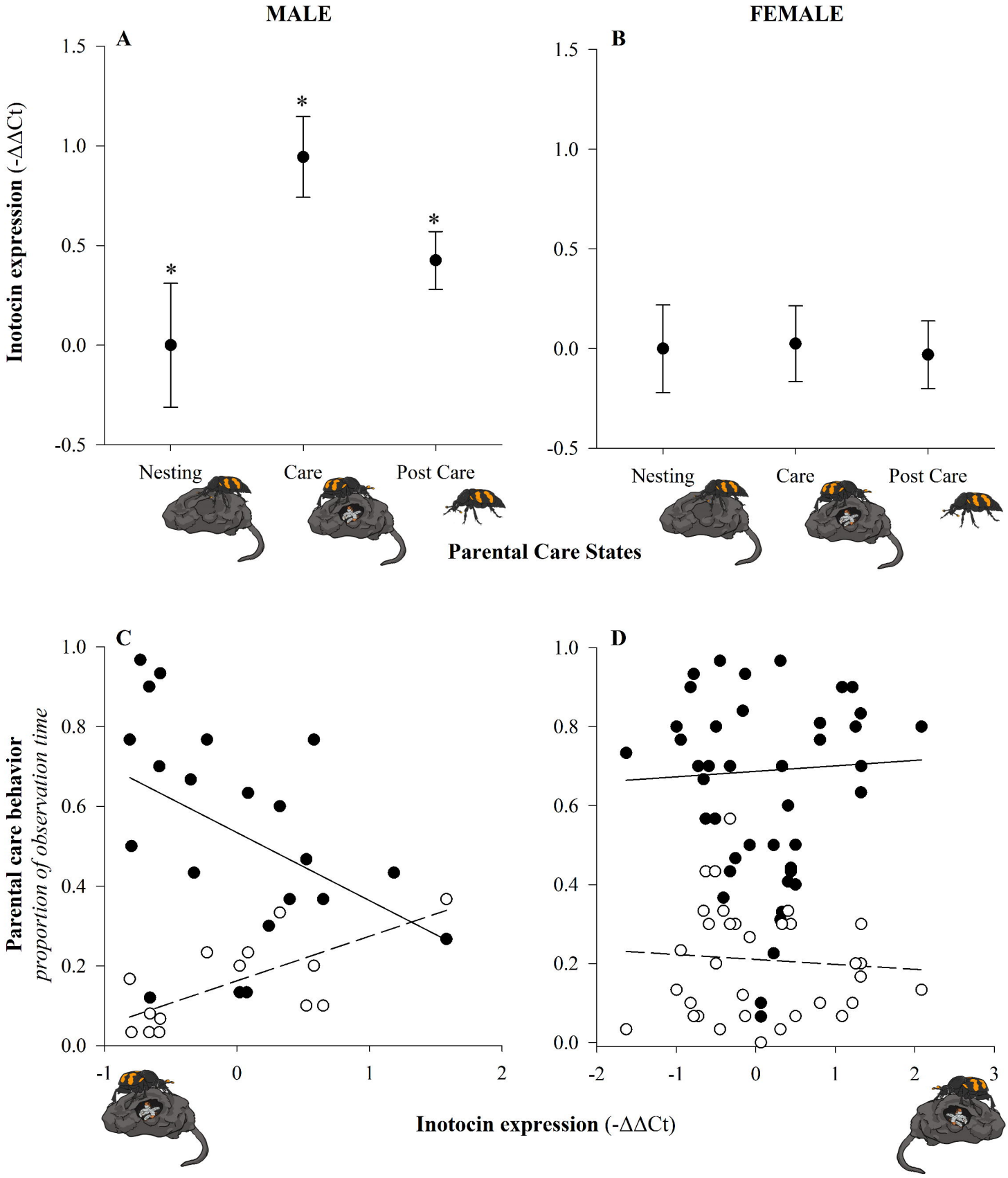
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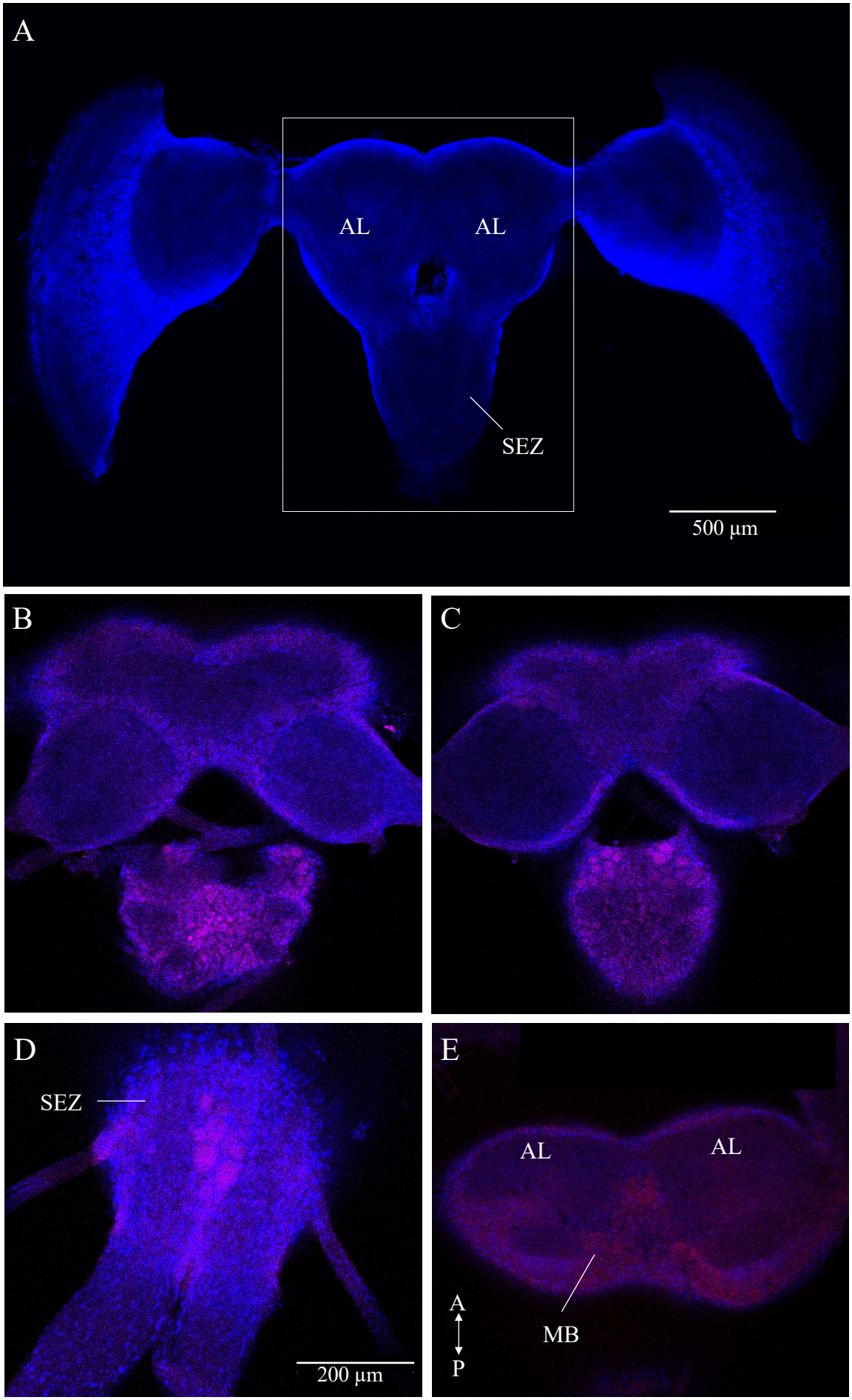
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