Oxytocin/vasopressin system of insects influences parental care in males but not females

Ahva L. Potticary*[1], Christopher B. Cunningham[1], Elizabeth C. McKinney[1], Patricia J. Moore[1], Amsale T. Belay[2], and Allen J. Moore[1]

[1]*Department of Entomology, University of Georgia, Athens, Georgia, 30602 USA*

[2]*Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, M5S 3B2 Canada*

***Corresponding author**

Ahva L. Potticary

Dept. of Entomology

120 Cedar Street

Biological Sciences Building #360

University of Georgia

Athens, GA 30602

+1.406.529.3416

ahva.potticary@uga.edu

ORCID identifier

Ahva L. Potticary: 0000-0002-1157-5315

Christopher B. Cunningham: 0000-0003-3965-2076

Patricia J. Moore:

Amsale T. Belay:

Allen J. Moore: 0000-0002-1498-3322

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Abstract

Gene duplication is thought to allow for the origin of novel gene functions. However, how duplication influences gene function is difficult to determine, as new adaptations may subsume previous traits and the ancestral state is often unknown. One solution is to investigate the functions of a gene that exists as a single and duplicated state in different species. Here, we leverage a system for which functions have been well-characterized in the duplicates – the oxytocin/vasopressin system in vertebrates – and that exists as a single copy – *inotocin* - in insects. To answer the question of how functions of a single copy are partitioned prior to gene duplication, we experimentally tested the relationship between *inotocin* gene expression and the known social and non-social functions of oxytocin/vasopressin in a subsocial beetle that expresses complex parental care. We found that *inotocin* was strongly related to water balance in both males and females, corroborating other evidence that water balance is an ancestral function. Moreover, we found that *inotocin* was associated with male, but not female, parental care states, influencing the expression of care behavior towards offspring in males. Lastly, using immunohistochemistry, we show that inotocin is broadly distributed in the brains of both female and male parents. These results suggest that the ancestral social function of the vasopressin system may have been to moderate flexibility in male, rather than female behavior, and the role of oxytocin/vasopressin in female-offspring interactions is more recent.

Significance Statement

Oxytocin/vasopressin are amongst the most studied neuropeptides in vertebrates, influencing water balance, mating interactions, and most notably, social bonding. This gene pair evolved from a duplication in the vertebrate lineage of an ancestral vasopressin-like gene. Are the multiple social effects in vertebrates due to this duplication, or are social influences also ancestral? Here, we demonstrate that, in a biparental social beetle with a single copy, inotocin influences social interactions between fathers and offspring as well as having the ancestral function of water balance in both males and females. In vertebrates, influences on social interactions with males is associated with both oxytocin and vasopressin. This may indicate that gene duplication allowed for the evolution of new behavioral functions in females.

Introduction

Gene duplication, or the production of two copies of an existing gene, is broadly thought to enable the evolution of gene function (1, 2). The fate of duplicated genes has been inferred in many ways, such as by using variation of function of recently duplicated genes (3), reconstructing the hypothetical sequence of the ancestor (4), and correlating the timing of whole genome duplication with the advent of new complex behavior (e.g., anadromy; (5)). Yet, determining the evolutionary fate of duplicated genes is challenging, as new adaptations can subsume previous traits and the ancestral state is often unknown, making it difficult to link historical and current gene functions. This is especially true for behavior, which often cannot be inferred from a fossil record. These constraints have hindered an understanding how behavior may evolve following gene duplication. One solution to this problem is to investigate the function of a gene that exists as a single copy in some species and as a duplicate in others.

Here, we leverage a system for functions have been well-characterized in the duplicates – the oxytocin/vasopressin system in vertebrates – and that exists in a single copy – inotocin - in insects. The nonapeptides oxytocin and vasopressin evolved from a tandem duplication event of a vasopressin-like gene (6-8) that occurred approximately 500 million years ago in the common ancestor of vertebrates (9), although the oxytocin and vasopressin group of homologs are part of an ancient family of conserved peptides that have existed for over 700 million years (10, 11). In vertebrates, oxytocin/vasopressin are best known for their influence on the regulation of complex social behaviors (such as parental care and pair bonding) and non-behavioral functions such as water balance and myoactivity (Fig. 1). Moreover, both peptides demonstrate great evolutionarily lability in how each function is influenced between males and females (SI Table 1; 10, 12-14), and oxytocin/vasopressin are often noted for their sex-specificity within species. For example, oxytocin and vasopressin can influence aggression in both sexes, yet there is not a consistent link between either peptide, the sex it acts in, and the expression of aggression across

vertebrate species (SI Table 1). However, while the sex-specificity and functions of oxytocin/vasopressin are well-understood, insects only possess a single homolog of oxytocin/vasopressin (15-18), providing a unique opportunity to determine how the observed functions of the duplicates (oxytocin/vasopressin) are influenced by a single homolog (inotocin). We particularly focus on the function of inotocin in males and females, as oxytocin/vasopressin are known to show sex-specificity in their function across vertebra taxa (SI Table 1). While some functions for inotocin are known (Fig. 1), it is unknown whether inotocin influences the expression of parental care.

To address these questions, we first reviewed the most highly conserved functions of oxytocin/vasopressin group in vertebrates and the sex in which they act, including water balance, parental and social behaviors (SI Table 1). We then investigated how the phenotypic functions of a single copy could be partitioned prior to gene duplication using the biparental subsocial beetle *Nicrophorus orbicollis*. Burying beetles require small animal carcasses to breed, where they perform extensive elaborate parental care. Upon finding a carcass, parents bury it, remove the exterior (e.g., fur), and coat it with an antimicrobial exudate (19). 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 (20). If larvae appear on the carcass too early, the breeding pair will not provide care to the offspring and will 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 does not involve social interactions). In general, males trade-off indirect care for direct care while females are consistent in their expression of both (21, 22). Larvae of *N. orbicollis* cannot survive without parental care and while females are highly consistent in their care, males show more environmentally dependent care behavior (e.g., loss of partner; 22, 23). Parental behavior in *N. orbicollis* thus requires nesting behavior, egg laying, a transition from infanticide to acceptance of larvae, and larval feeding, concordant with the behavioral functions of oxytocin/vasopressin in vertebrates (Fig. 1).

We leveraged this variation across the lifetime of *N. orbicollis* to address whether *inotocin* and *inotocin receptor* gene expression are associated with the functions of oxytocin/vasopressin in vertebrates. *inotocin* and *inotocin receptor* were related to water balance in both males and females, corroborating other work indicating that water balance was one ancestral function of this neuropeptide group. Isolated male virgins showed an increase in *inotocin* and *inotocin receptor*, corresponding to the age when males have reached sexual maturity. Moreover, we found that *inotocin* was related to parental care behavior in males but not in females. Males that were actively caring for larvae had higher *inotocin* expression than males than males without larvae. Males that spent more time feeding their larvae had higher *inotocin* than males that fed their larvae less, and higher *inotocin* than males that spent more time engaged in indirect (non-social) parental care behaviors. Lastly, immunohistochemistry reveals that inotocin is broadly expressed in the brains of both male and female parents. Together, these results may suggest that duplication of an ancestral vasopressin-like neuropeptide allowed for neofunctionalization of the duplicate (oxytocin) and the subsequent elaboration of novel gene function in females.

Results

Inotocin Associated with Non-social Contexts.

In females, neither the expression of *inotocin* ($F_{5,54}$ = 1.27, P = 0.30; Fig. 2a) nor *inotocin receptor* ($F_{5,54}$ = 1.44, P = 0.22; Fig. 2A) changed with adult age. In contrast, in virgin males, *inotocin* ($F_{5,54}$ = 3.45, P = 0.009; Fig. 2B) and *inotocin receptor* ($F_{5,54}$ = 3.90, P = 0.004; Fig. 2B) expression both changed across this timespan. This difference was primarily driven by lower *inotocin* and *inotocin receptor* expression in 6-day old males relative to post-reproductive ages (day 21 for *inotocin* and days 21 and 35 for *inotocin receptor*, Tukey's HSD; Fig. 2B). A trend of lower expression of *inotocin* and *inotocin receptor* was seen

in both males and females in day 6, but the differences were not large in either sex. Six days is prior to sexual maturity in both sexes.

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. Female beetles in Low Humidity treatments had trend towards higher *inotocin* ($t_{1,18}$ = -1.96, P < 0.07; Fig. 2C), while Low Humidity females expressed higher *inotocin receptor* ($t_{1,18}$ = -2.12, P < 0.05; Fig. 2C), than females in the High Humidity treatment. 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. 2D).

Inotocin in Social Contexts.

Females that accepted and cared for larvae did not differ in *inotocin* or *inotocin receptor* expression relative to females that committed infanticide (*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. 3A) or *inotocin receptor* expression ($F_{2,57}$ = 0.21, P = 0.81). 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). There was no relationship between the proportion of time females spent feeding larva and *inotocin* or *inotocin receptor* expression (*inotocin*: $F_{1,32}$ = 0.17, r^2 = 0.005, P = 0.69, Fig. 3B; *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. 3B) or *inotocin receptor* ($F_{1,33}$ = 0.0004, r^2 < 0. 0001, P = 0.98).

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). However, 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. 3c). 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). Moreover, males that spent more time feeding larva had higher *inotocin* expression than males that fed less ($F_{1,14}$ = 8.91, r^2 = 0.39, P < 0.001; Fig. 3d). There was no relationship between the amount of time males spent feeding larva and *inotocin receptor* ($F_{1,14}$ = 0.08, r^2 = 0.005, P = 0.79). Amount of indirect care provided was negatively associated with *inotocin* expression in males ($F_{1,19}$ = 4.33, r^2 = 0.19, P = 0.05; Fig. 3d), but again there was no relationship between *inotocin receptor* and amount of indirect care by males ($F_{1,19}$ = 0.06, r^2 = 0.003, P = 0.80).

Immunohistochemistry of inotocin in the brains of parents with larva showed diffused expression in both male and females (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 major differences observed in either the pattern or density of staining in male versus female brains.

Discussion

Our results demonstrate that inotocin in *N. orbicollis* is associated with both non-social and social contexts. Consistent with the water balance function of oxytocin/vasopressin across broad taxonomic groups, we also found that *inotocin* is involved in water balance in both sexes (Fig. 1, SI Table 1). This supports other research indicating that water balance was an ancestral function of the oxytocin/vasopressin system (24, 25). However, the discovery that *inotocin* expression changes as beetles age in isolated, male virgins, but not females, was unexpected (Fig. 2). In males, pre-reproductive males had lower inotocin expression than post-reproductive stages. It is possible that *inotocin* is involved in

priming males for reproduction, but further research would be needed to determine how the development of inotocinergic system may be related to reproduction.

Given the subsocial and complex parental care observed in this species, we predicted a social role for inotocin as well. However, rather than a general role in parental care, we found that increased *inotocin* expression was associated with male, but not female, parenting in *N. orbicollis*. This holds for both transition from nesting to parental offspring provisioning, 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 social associations of *inotocin* did not hold for females. Overall willingness to accept or commit infanticide of larvae was not associated with differences in *inotocin* or *inotocin receptor* expression in either males or females, suggesting that inotocin has a role limited to social interactions involved in male parenting behavior. Despite this, immunohistochemistry revealed that brains of parenting males and females showed similar patterns of expression, with the majority of staining occurring in the suboesophageal zone (SEZ) and the mushroom bodies (MB; Fig. 4).

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 social interactions involving parenting arose de-novo in burying beetles. This is possible but not parsimonious given the distribution of social influences of oxytocin/vasopressin homologs across vertebrate taxa (SI Table 1). Second, given both males and female social interactions 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 other studies of female insects have not found a definitive influence of inotocin on parenting behavior (26, 27). Finally, it may be that influences on male rather than female social behavior is ancestral. A similar, male-specific role was found in *nematocin*, the single copy homolog for oxytocin/vasopressin found in nematodes, where *nematocin* was found to function in the organization of male reproductive behavior but not females (28). It is possible that *inotocin* has a similar function in males, and acts to organize male parental care behavior or increase salience of offspring cues, and as parental care is often far more variable in males than in females, the effects are only apparent in males.

Oxytocin/vasopressin have been found to increase the salience of social signals and group formation in a context-dependent manner (29, 30), and are associated with plasticity in socially-mediated behaviors (e.g., 31). In many insects that have parental care, there is little plasticity in 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. In contrast, male burying beetles tend to be very flexible in care whilst females are more canalized (21, 22). It is possible that, in *N. orbicollis*, *inotocin* functions to draw male attention to offspring, but only under specific conditions. Support for this idea is provided by the results that males with higher *inotocin* fed their larva more (i.e., increased attention to larva) 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 larva. This hints that there is some threshold of *inotocin* expression that facilitates a transition from other kinds of care duties – e.g., nest defense, carcass preparation – to direct care of larva, although future research is needed to disentangle this effect.

Immunohistochemistry shows diffuse staining for inotocin expression across the brains of both males and female parents (Fig. 4), which does not exclude a social role for inotocin in females. Previous work has indicated that inotocin is often produced in the SEZ, and inotocin in this location can be related to water balance pathways (18, 27). Mushroom bodies have been associated with social behavior in insects more broadly (32, 33), 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 behavior is influenced. However, future research would be needed to determine how the distribution of inotocin influences social behavior in males and females.

Oxytocin was first discovered in female vertebrates in association with parturition (34), and correspondingly, there has been a greater emphasis on the role of oxytocin in females with a lesser emphasis on vasopressin and males (SI Table 1). For this reason, the discovery that an ancestral proxy functions in male parental care behavior, but not female parental care, is contrary to initial expectations for most researchers. Nevertheless, the results of this study support the idea that male influences are

ancestral, and that duplication of an ancestral vasopressin-like neuropeptide in vertebrates allowed for neofunctionalization of the duplicate the elaboration and evolution of behavioral functions in females. Multiple lines of evidence support the idea that the ancestral gene was more similar to *vasopressin* (6, 8) and even *inotocin* is more structurally similar to the *vasopressin* group than *oxytocin*, despite its name (17). The oxytocin/vasopressin system exhibits high levels of plasticity, sexual dimorphism, and species-specificity across taxa (SI Table 1), hinting at sexually dimorphic functions of the ancestral peptide.

Methods

Insect Colony and Husbandry.

The *Nicrophorus orbicollis* used in this study were part of an outbred colony that is regularly supplemented with wild-caught beetles from Whitehall Forest in Athens, GA, USA. All beetles are maintained 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. All individuals used for this study were bred in the laboratory to ensure that parentage, age, and rearing conditions were known and standardized. 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 larvae are placed 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 behavioral or physiological treatments. Individuals were defined as age Day 1 on the day of eclosion. Beetles were fed *ad libitum* organic ground beef twice a week following eclosion.

Inotocin and Non-social Contexts.

Age Series.

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). The post-eclosion individuals were collected 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 (18, 35). This age series corresponds to the timing when beetles were collected for each behavior state and the water balance experiment (SI Fig. 1).

Water Balance.

To determine the relative humidity range that would be expected to induce water balance, we first 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, using a handheld digital Preciva Humdity and Temperature Reader (model no. HT154001). 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, individuals were transferred to an individual container with approximately 2 cm of potting soil with perforations nested inside of a breeding box and sealed with parafilm. Low and High humidity conditions were simulated 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 36). 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 (36). For this reason, beetles were left in Low Humidity or High Humidity treatments for 8 hours to provide sufficient time for water stress to occur and to ensure that beetles survived until the end of the treatment period. To account for the fact that burying beetles experiencing water stress may bury themselves in soil to evade low humidity, individuals in the Low Humidity experiment were kept in individual containers without soil to ensure that they could not mitigate exposure by burrowing into the soil. The relative humidity and temperature of each set-up was measured at the end of the 8-hour period.

Inotocin and Social Behavior.

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. The pair was then moved 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.). Breeding boxes were returned to the incubator and kept on a darkened shelf. Breeding boxes were checked for eggs every twenty-four hours. Once eggs were observed, the focal female and the brood ball were moved 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 returned to the breeding box with the female. Once the male and female were reunited, breeding boxes were again checked daily for eggs.

Affiliation and Infanticide.

To test the hypothesis that *inotocin* influences the transition from aggression to affiliation towards larvae, we compared males that committed infanticide with those that accepted and cared for larvae arriving on the mouse resource. *Nicrophorus* species identify their offspring using temporal cues, such that larva that arrive too early on the carcass are eaten and those that arrive on time are accepted by the parents and fed

(20). These treatments took advantage of this temporal recognition to sample adults as they committed infanticide of the larva or accepted and fed offspring, respectively. For both treatments, the breeding pair were provided with ten larvae, at least 5 of which were their offspring from the first clutch of eggs and were supplemented with same-age larva from other clutches if insufficient numbers were available from the focal pair's clutch. Following the addition of larvae, the focal individuals was observed for at least thirty minutes to detect acceptance or infanticide of larva. If the focal individuals did not respond in this time, pairs were checked every 30 minutes to identify the onset of acceptance or infanticide. In some cases, one parent would accept larva while the other parent committed infanticide; in these cases, the male and female were sampled and categorized separately according to the phenotype that was observed for each individual. For the treatment where the male committed infanticide, focal individuals were collected while the focal individual was attacking/eating larvae. For the treatment where larvae were accepted, focal individuals were collected immediately after the parent made antennal contact with the larva 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 (20) and males likely base their window on timing and frequency of female acceptance of mating (unpub. data). Thus, to reach sample size, most males in the treatment where larvae were accepted were moved with the brood ball and females were removed after the first batch of eggs were laid, which increased the likelihood of male acceptance of larva.

Parental Care.

To test the hypothesis that *inotocin* or *inotocin receptor* influences 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).

For the Nesting treatment, we collected whole heads of males and females 24-36 hours after the male had been reunited with the female. For individuals in the Care treatment, eggs from the second clutch were allowed to hatch and crawl to the brood ball naturally to ensure female acceptance of larva. Each breeding box was checked every 12-24 hours to determine when larvae arrived on the brood ball. The pair was allowed to interact with larvae for 24 hours, and then parental care behavior was scored during a behavioral observation (see below *Behavioral observations*). Whole heads of the focal male and female were collected 24 hours after the larva 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 behavior. Male parental care is variable and often depends on the presence and activity of the female (23, 37). For males that did not show direct parental care during the initial behavioral observation, females were removed to induce male parental care, and then males were collected 24 hours later while 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 behavior, the male and female were housed 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 behavior 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, the pair was allowed to breed normally and were monitored every 24 hours to determine egg lay date and the arrival of larvae. Twenty-four hours after hatch, the pair was observed for 30-minutes and whole heads were collected as they were engaged in direct care behavior (approximately Day 35). Similar to the observations performed for Care treatments, if a male did not show direct care behavior during the initial behavioral observation, the female was removed for collection and males were collected 24 hours later while engaged in direct care behavior. When mortality

of one parent occurred during or immediately after first breeding attempt, a new mate was provided to the focal individual (n = 7 of 20 breeding attempts).

Behavioral Observations of Parental Care.

We conducted focal behavioral 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. All observations were conducted under red lights to ensure no light effects on individual care behavior, as *N. orbicollis* are nocturnal (38). In each behavioral observation, we recorded both direct and indirect care (37). 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. Time spent showing "no care" was also recorded, and is defined as when the parent was on the carcass but not engaged in direct or indirect care, or was off of the carcass. Behaviors were recorded as a frequency (e.g., number of feedings) and a duration (length of time engaged in the behavior). These data were collapsed to align with previous research using instantaneous 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. This method was used because specific behaviors are often difficult to disentangle, e.g., larval feeding is often interrupted by the adults "self-feeding", which is often followed by direct provisioning of larvae. For this reason, continuous observation allowed for the observer to categorize behavior into direct or indirect care by placing the behavior at each time point into its functional context. For males that did not show either indirect or direct parental care during the behavioral observations (n = 13), the female was collected and removed to initiate care behavior in the males. For these males, direct care of larvae was verified prior to collection by observing at least one larval feeding event.

RNA Sample Collection and Analysis.

Whole Head Collection.

We collected samples with dissecting scissors, flash freezing them in liquid nitrogen (39), 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.

Gene Expression Analysis for Inotocin and Inotocin Receptor.

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 (39), 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. (40) and using transcriptome of Benowitz et al. (41). 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 manufacture'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.

Immunohistochemistry of Inotocin in the Brain.

We dissected brains from female and male beetles at the period of peak parental care behavior, 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. Brains were then rinsed 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.

The brains were incubated in a 1:300 dilution of primary polyclonal antibody that recognizes the pre-pro-peptide of inotocin (27) 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 in order 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. Brains were then mounted on depression well slides and imaged 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).

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 (42) to convert raw expression data to normalized relative expression values. For all analyses, males were used as the comparison group for male analyses, and females were used as the comparison group for female analyses. For the age series, virgin D21 were used as the control group. For all behavioral states (Accept vs. Infanticide, Nesting, Care, and Post Care, and Care vs. Double Care) we used the behavioral state that occurred earliest within the experiment as the comparison group (Infanticide, Nesting, and Care, respectively). For the Low vs. High Humidity experiment, we used High Humidity as the comparison group. 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 determined the direction of significance for age analyses using post hoc Tukey's HSD test.

We also assessed if there was a direct association between *inotocin* and *inotocin receptor* expression and parental care behaviors observed in behavioral observations. Data from individuals in the Care and Double Care treatments were pooled as there was no difference in *inotocin* and *inotocin receptor* expression between treatments (see Results). The influence of *inotocin* and *inotocin receptor* expression on care behavior (proportion of observation points spent in direct care) was analyzed using linear regression for individuals that were observed to feed larva at least once during the targeted

behavioral observation without removing the mate (females: n = 34, males: n = 16). The influence of *inotocin* and *inotocin receptor* expression on indirect parental care was analyzed using linear regression for individuals that interacted with the brood ball or larvae in any way (females: n = 34, males: n = 21). For one behavioral observation, the pair buried the carcass and the observation time was thus only 25 minutes; for this reason, all behavioral data are presented as a proportion of the observation time. A subset of observations was excluded (n = 6) because male and female behavior was more indicative of a response to perceived competition or disruption (i.e., frequent stridulation and both individuals off of the carcass exploring breeding box).

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Figure legends

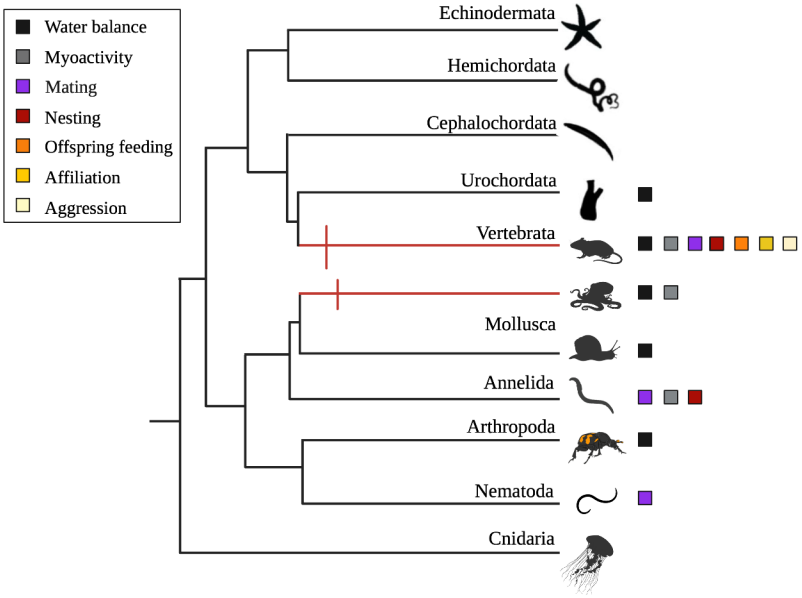
Figure 1. Oxytocin/vasopressin family evolved from duplication event. An ancestral vasopressin-like neuropeptide was duplicated in the common ancestor of jawed vertebrates, and another duplication occurred in the cephalopods. Duplication events are denoted by a red line. Neuropeptides of the oxytocin/vasopressin 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 homologs are indicated next to the taxonomic group they function in. 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 (43) in BioRender.

Figure 2. Expression of *inotocin* and *inotocin receptor* genes is associated with non-social contexts. Orange circles represent *inotocin* expression, while closed circles represent *inotocin receptor*. (***A***) *Inotocin* and *inotocin receptor* does not change across the lifetime of females, 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; this difference was primarily driven by lower *inotocin* and *inotocin receptor* expression in 6-day old males relative to post-reproductive ages (letters above bars indicate significantly different expression levels between ages). Lastly, (***D***) *inotocin* and *inotocin receptor* were

both associated with water balance in males. For *B* & *D*, p-values are denoted over the lines, with a P-value less than 0.05 (*), and less than 0.01 (**). Results are mean ± SEM (n = 10 for each treatment).

Figure 3. Inotocin associated with male but not female parental care. (***A***) There was no difference in *inotocin* for females across parental care states (n = 20 for each state), nor was there (***B***) a relationship between *inotocin* and the proportion of time females spent either feeding larva (open circles and dashed line) or engaging in indirect care (closed circles and solid line; n = 34). On the other hand, (***C***) males did show an association between parental care states and *inotocin* (n = 20 for each), and (***D***) for the subset of males that performed care behavior 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. For *A* & *C*, significant P-values are denoted over the lines, with a p-value less than 0.05 denoted*. Results are mean ± SEM.

Figure 4. Distribution of inotocin in the brain. (***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 also 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 ventral (V) to dorsal (D).


Water balance
Myoactivity
Mating
Nesting
Offspring feeding
Affiliation
Aggression
Echinodermata
Hemichordata
Cephalochordata
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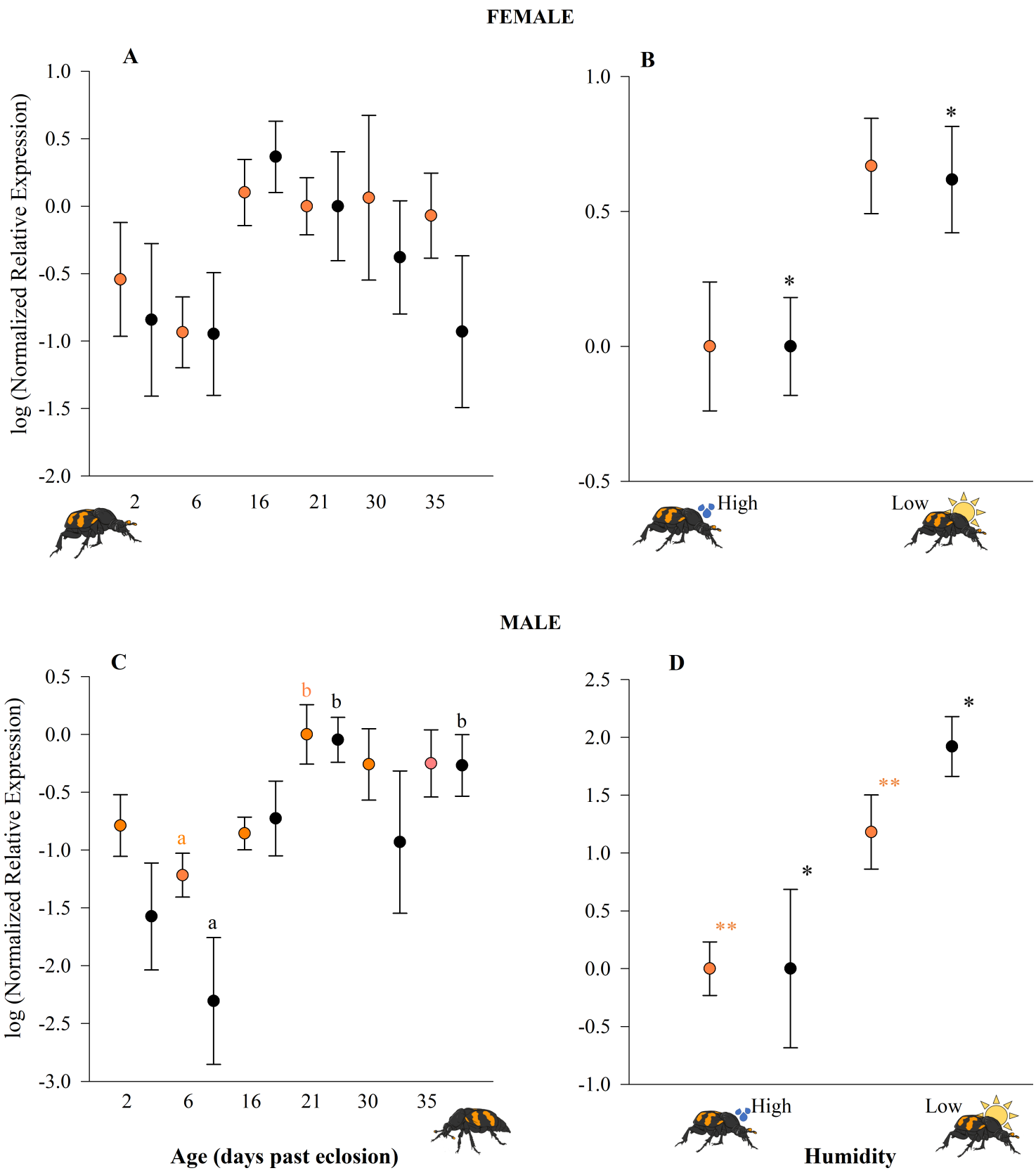
Vertebrata
Mollusca
Annelida
Arthropoda
Nematoda
Cnidaria


FEMALE
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B
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0.0
-0.5
-1.0
-1.5
-2.0
2
6
16
21
30
35
*
*
High
Low
MALE
C
D
log (Normalized Relative Expression)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
a
a
b
b
b
2.5
2.0
1.5
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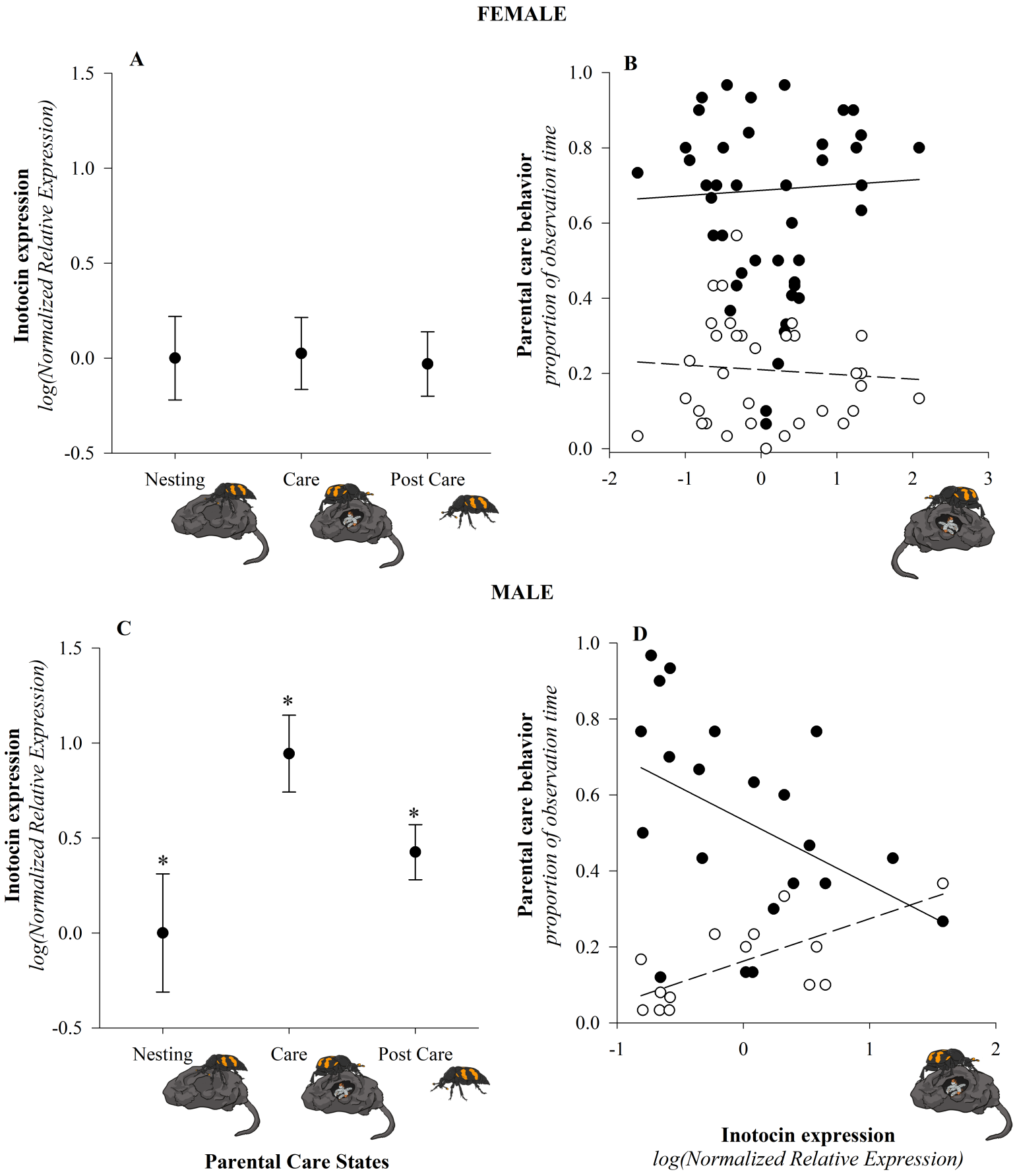
Age (days past eclosion)
Humidity


FEMALE
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Inotocin expression
log(Normalized Relative Expression)
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-0.5
Nesting
Care
Post Care
B
Parental care behavior
proportion of observation time
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0.8
0.6
0.4
0.2
0.0
-2
-1
0
1
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3
MALE
C
Inotocin expression
log(Normalized Relative Expression)
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1.0
0.5
0.0
-0.5
*
*
*
Nesting
Care
Post Care
Parental Care States
D
Parental care behavior
proportion of observation time
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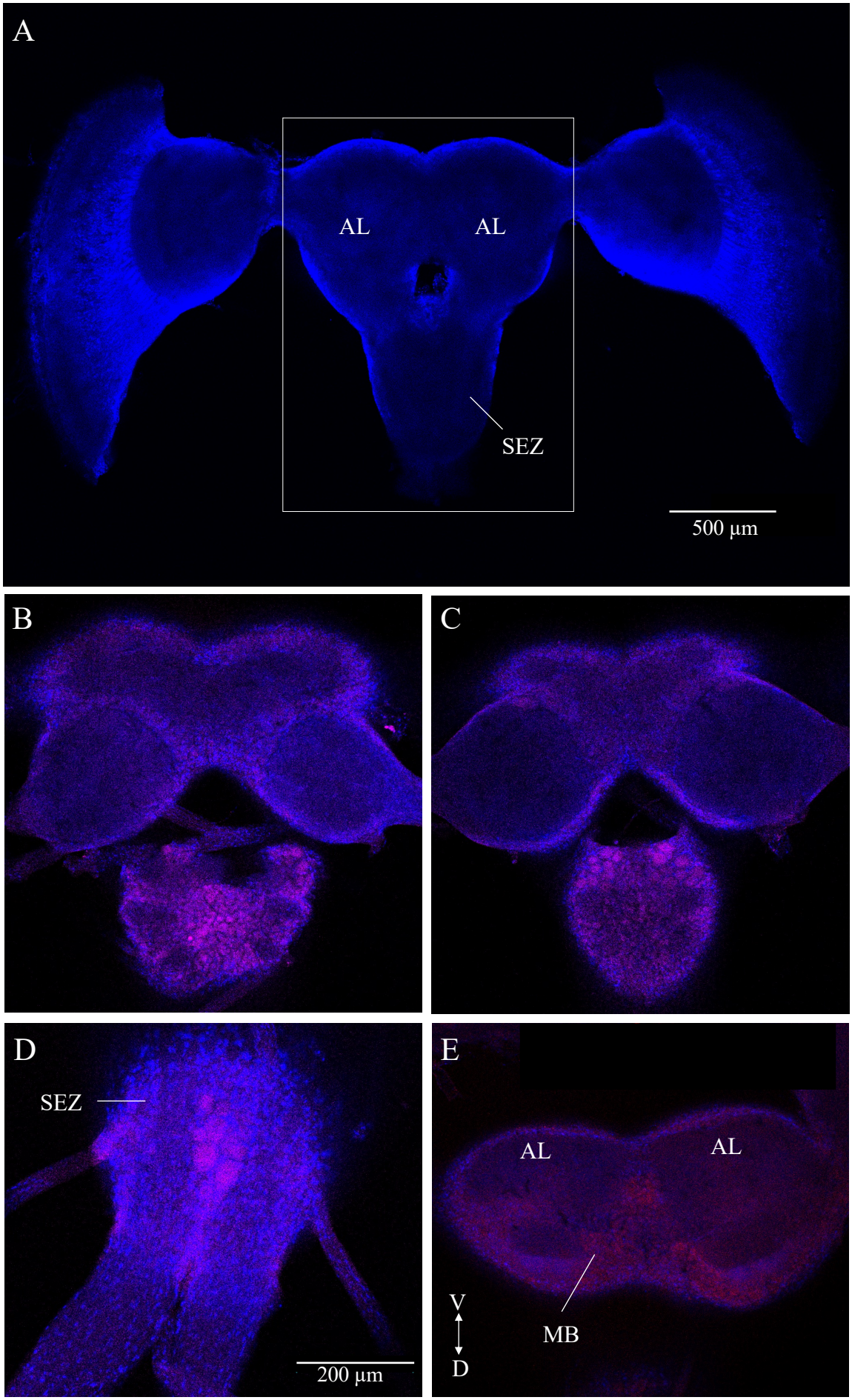
Inotocin expression
log(Normalized Relative Expression)


A
AL
AL
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500 µm
B
C
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200 µm
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AL
AL
MB
V
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