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# Biparental behavior in the burying beetle *Nicrophorus orbicollis*: a role for dopamine?

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## Abstract

Burying beetles *Nicrophorus orbicollis* exhibit facultative biparental care of young. To reproduce, a male–female burying beetle pair bury and prepare a small vertebrate carcass as food for its altricial young. During a breeding bout, male and female behavior changes synchronously at appropriate times and is coordinated to provide effective care for offspring. Although the ecological and evolutionary factors that shape this remarkable reproductive plasticity are well characterized, the neuromodulation of parental behavior is poorly understood. Juvenile hormone levels rise dramatically at the time beetle parents accept and feed larvae, remain highly elevated during the stages of most active care and fall abruptly when care is terminated. However, hormonal fluctuations alone cannot account for this elaborate control of reproduction. The biogenic amines octopamine (OA), dopamine (DA), and serotonin (5-HT) mediate a diversity of insect reproductive and social behaviors. In this study, we measured whole brain monoamine levels in individual male and female burying beetles and compared OA, DA, and 5-HT profiles between breeding (parental) and nonbreeding, unmated beetles. Remarkably, after 24 h of care, when parental feeding rates begin to peak, DA brain levels increase in breeding beetles when compared to nonbreeding controls. In contrast, brain OA and 5-HT levels did not change significantly. These results provide the first evidence for a potential role of DA in the modulation of burying beetle parental behavior.

**Key words:** dopamine, *Nicrophorus*, neuromodulation, octopamine, parental care, serotonin.

Extended biparental care is a reproductive strategy that is rarely exhibited by insects or by most vertebrate species. Among vertebrates, biparental care is ubiquitous in birds but unusual in mammals, with primates and carnivores, for example, serving as notable exceptions (Clutton-Brock 1991). In insects, utilization of rich and ephemeral reproductive resources like dung or carrion is thought to have promoted selection for unique behavioral and physiological adaptations leading to subsociality and biparental behavior (Tallamy and Wood 1986). Given the complex underpinnings of caregiving, the study of parental care can provide critical insights into the interplay among ecological, social, and neuroendocrine factors in the regulation of behavior. The pervasive roles of hormones in the control of reproductive social behavior have been explored extensively in vertebrates

(Ketterson and Nolan 1992; Buntin 1996; Adkins-Regan 2005). By comparison, many aspects of reproductive behavior and physiology, including the hormonal basis of parental care, remain poorly understood in insects (Trumbo 2002; Riddiford 2012). Remarkably, the neuroendocrine system of insects bears fundamental similarities to the hypothalamo–hypophyseal axis of vertebrates (Scharer 1987; Hartenstein 2006; Wirmer et al. 2012), further opening a unique avenue for comparative studies of the physiology of reproduction.

The burying beetle, *Nicrophorus orbicollis*, provides an experimentally accessible model for studies of insect parental care (Trumbo 1996, 2012). The natural history of burying beetles is well described (Pukowski 1933) and the ecological and evolutionary processes that have contributed to molding their intricate reproductive behaviors are

well understood (Trumbo 1996, 2012; Eggert and Müller 1997; Scott 1998). During reproduction, a male–female beetle pair will cooperate in burying and preparing a small vertebrate carcass as food for its altricial larvae. Upon discovery, the carcass is quickly concealed underground, fur or feathers removed and the carrion mass rolled into a ball. Within 48 h following carcass discovery, the female lays eggs in the soil nearby and 3–4 days later larvae hatch and make their way to the carrion ball. Larvae beg and are fed, typically by both parents, regurgitated predigested food. In *N. orbicollis*, the offspring are entirely dependent on parental regurgitations for survival soon after hatching (Trumbo 1992). During a breeding bout, male and female behavior has to change at appropriate times, from passive to aggressive responses to conspecific intruders, and from infanticidal to parental, a few hours before larvae hatch and arrive on the carcass (Müller and Eggert 1990; Scott 1990; Oldekop et al. 2007). Male and female burying beetles also need to coordinate their reproductive tasks during larval care, the most demanding stage of the reproductive cycle (Fetherston et al. 1990). Parental activities center on provisioning and protecting the larvae, maintaining the brood chamber, as well as processing and treating the carcass with antimicrobial secretions to delay its decomposition (Fetherston et al. 1990; Robertson 1993; Scott 1998; Arce et al. 2012).

While many of the costs and benefits associated with reproductive cooperation and conflict have been thoroughly explored, both in the field and in the laboratory (Scott 1998), we have still an incomplete understanding of the physiological factors, neural circuits, or molecular pathways involved in promoting the highly plastic reproductive and parental behaviors of burying beetles. In previous work, we found that in *N. orbicollis*, hemolymph titers of juvenile hormone (JH) rise dramatically at the onset of parental behavior, are very high during the most active stages of larval care when rates of parental feedings peak, and decline rapidly before care is terminated (Panaitof et al. 2004). Importantly, in both males and females, JH levels are responsive to social cues, such as the presence or absence of a mate and brood age or size (Panaitof et al. 2004; Scott and Panaitof 2004). Despite the remarkable correlation between changes in circulating JH levels and the well-timed behavioral shifts that male and female burying beetles undergo during reproduction, the precise neurophysiological mechanisms responsible for the initiation and maintenance of care remain elusive. Direct treatment with fluvastatin, a robust inhibitor of JH biosynthesis (Debernard et al. 1994), while significantly lowering JH levels (Panaitof and Scott 2006), does not seem to interfere with either the acceptance of larvae or initiation of parental feedings (Panaitof 2006). Thus, it is unclear whether JH has a direct or an indirect role in the transition to parental behavior and initiation of care. This raises the likely possibility that JH may act in conjunction with other unidentified neuromodulatory pathways. Lending support to this idea, several studies in the eusocial Hymenoptera have shown that JH interacts with the biogenic amines octopamine (OA) and dopamine (DA) in the regulation of reproductive maturation, behavioral development, and age-related task specialization in honey bee workers (Schulz et al. 2002a, 2002b; Harano et al. 2008; Sasaki et al. 2012a). Moreover, treatment with the JH analog, methoprene, causes elevation of OA and DA levels in the honey bee brain (Schulz et al. 2002a, 2002b; Harano et al. 2008; Sasaki et al. 2012a).

Based on their wide involvement in a variety of invertebrate reproductive and social behaviors (Roeder 1999, 2005), combined with evidence that JH alters monoaminergic function (Harano et al. 2008; Sasaki et al. 2012a), we hypothesized that the biogenic amines OA, an analog of norepinephrine in vertebrates (Roeder 2005), DA

and serotonin (5-hydroxytryptamine; 5-HT), represent the most likely candidates for contributing to the neuromodulatory control of parental behavior in the burying beetles. Since the discovery of their synthesis and release by the central nervous system of insects, the diverse functions of biogenic amines and their receptor subtypes have received considerable interest (Roeder 1999, 2005; Farooqui 2012), with functional studies revealing that the invertebrate octopaminergic system shares fundamental properties with the adrenergic system of vertebrates.

Variation in OA levels has been implicated in the modulation of behavioral state in a number of insects. In the highly eusocial honey bees *Apis mellifera*, rising OA levels are linked to behavioral maturation and age-related division of labor, illustrated by the transition from nursing (brood care) to foraging activities (Wagener-Hulme et al. 1999; Schulz and Robinson 2001; Schulz et al. 2002a, 2002b). Brain OA levels of foragers are higher than those of nurses (Wagener-Hulme et al. 1999; Schulz et al. 2002a, 2002b) and OA treatment results in precocious expression of foraging behavior in honey bee workers (Schulz and Robinson, 2001; Schulz et al. 2002a, 2002b). Octopamine also facilitates nestmate recognition (Robinson et al. 1999) and has been similarly implicated in the discrimination of foraging-related stimuli and olfactory learning (Hammer and Menzel 1998; Barron et al. 2002; Scheiner et al. 2002). Recent studies of the octopaminergic system in the burying beetle, *Nicrophorus vespilloides*, show differential gene expression of several OA receptor subtypes across reproductive and social contexts thus linking OA signaling to behavioral flexibility during breeding (Cunningham et al. 2014).

Biogenic amines have also been implicated in the modulation of aggression in a number of invertebrate models (Kravitz and Huber 2003; Stevenson and Schildberger 2013; Alekseyenko and Kravitz 2014; Bubak et al. 2014a). The roles of OA, the invertebrate “fight or flight” neurotransmitter, and 5-HT have been extensively explored, especially in the social context-dependent initiation and escalation of fighting behavior and establishment of dominance. In crustaceans, 5-HT was repeatedly linked to aggression motivation, presumably by altering the behavioral threshold for retreat (Huber et al. 1997; Kravitz 1988, 2000). In territorial nonsocial insects like the cricket, *Gryllus bimaculatus*, OA acts to restore fighting readiness following defeat (Hofmann and Stevenson 2000) and 5-HT has been functionally linked to the modulation of fighting behavior and expression of behavioral features associated with dominant social status (Dyakonova and Krushinsky 2013). The genetic tractability of the fruit fly, *Drosophila melanogaster*, has further enabled remarkable dissection of neural circuits and neuromodulatory pathways involving both the serotonergic and dopaminergic system in the initiation and ability to escalate aggressive behavior (Zwarts et al. 2012; Alekseyenko et al. 2013; Alekseyenko et al. 2014). Novel model systems of invertebrate aggression, such as the stalk-eyed fly, *Teleopsis dalmanni*, have also provided important additional insights into the role of 5-HT in mediating conflict escalation, contest behavioral outcome and establishment of a winner–loser relationship (Bubak et al. 2014a, 2014b).

Given the robust links established between OA, 5-HT, and DA and the modulation of behavioral state, we sought to similarly explore whether the levels of the three monoamines change significantly as a result of the transition from nonparental to parental state in the burying beetles. Burying beetles, in addition to exhibiting behavioral changes toward newly hatched and developing larvae, including acceptance (decrease in aggression) and provisioning of larvae, also show changes in aggression toward adult conspecifics as

a means of protecting the offspring (Müller and Eggert 1990; Scott 1990; Robertson 1993). Thus, it seems likely that changes in central monoaminergic function associated with changes in aggression and/or motivation to perform parental tasks would be involved in modulating the response of burying beetles to social cues from competitors or intruders, mates, and young during breeding.

In the current study, we test a potential role for central monoamines in modulating burying beetle parental behavior by measuring whole brain levels of OA, 5-HT, and DA in male and female burying beetles prebreeding and during the breeding cycle, 24 h after the initiation of care. We predicted that the behavioral transitions associated with the acceptance and feeding of larvae for 24 h would be accompanied by significant changes in the brain levels of OA, 5-HT, and DA in breeding animals but not in nonbreeding controls. We interpret these findings in the context of exploring a neuromodulatory mechanism that may mediate the behavioral shifts associated with the onset of care and organization of parental activities in the burying beetles during breeding.

## Materials and Methods

### Animals and breeding experiments

Experimental animals were laboratory-reared offspring of wild-caught beetles trapped in Buffalo County, NE, in May–June 2014. Beetles were housed in plastic boxes lined with damp paper towels, with up to 10 same-sex individuals, and maintained at 20 °C on a 14L:10D cycle. They were fed a diet of beef or chicken liver *ad libitum*. All animals were previously unmated, sexually mature, ranging in age from 21 to 30 days at the time of the experiments. To initiate breeding, at “lights-off” each male and female was randomly assigned to a breeding pair ( $N = 12$  pairs) and provided with a 20–25 g previously frozen mouse carcass (RodentPro.com, Inglefield, IN) in a box ( $19 \times 14 \times 10$  cm<sup>3</sup>) three-fourth filled with peat moss. Each box was monitored for the presence of eggs and the time of oviposition was recorded for each beetle pair. Eggs were monitored daily until larvae hatched (typically 3–4 days later). The timing of larvae arrival on the carcass was recorded for each pair. The behavior of each beetle pair was then monitored until the first parental feeding of larvae was observed. We chose to evaluate central monoamine levels following 24 hours of care, given that parental feeding activities are highest at this stage (Fetherston et al. 1994). Twenty-four hours following the initiation of parental feedings, each beetle pair and brood chamber were briefly observed to ensure that larvae had been fed and developed to second instar stage.

### Brain dissection and HPLC analysis

Twenty-four hours after the onset of parental care, the male and female of each pair were sacrificed and the brains were dissected in ice-cold insect saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH = 7.4) and transferred to an acetate buffer (pH = 5.0; 60 µL) containing the internal standard  $\alpha$ -methyl DA ( $1 \times 10^{-7}$  M). Individual male and female brains of nonbreeding (control) beetles from the same colony were similarly obtained in the same experimental session. The brains were lightly disrupted by sonication, frozen, then thawed, and centrifuged ( $17,000 \times g$ , 4 °C). The monoamines were separated using a NOVAPAK C18 column (Waters Associates, Inc.) and detected using an LC 4 potentiostat and a glassy carbon electrode (Bioanalytical Systems) set at 0.5 nA/V with an applied potential of +0.997 V versus an Ag/AgCl reference electrode (Bubak et al.

2013). The pellet was solubilized in 0.4 M NaOH and analyzed for protein using the Bradford method (Bradford 1976). Monoamine concentrations were determined using a CSW32 data program (DataApex, Prague, Czech Republic) set in internal standard mode using peak height values relative to standards. The resulting concentrations were divided by µg protein in the sample yielding pg amine/µg protein and corrected for injection versus preparation volume.

### Data analysis

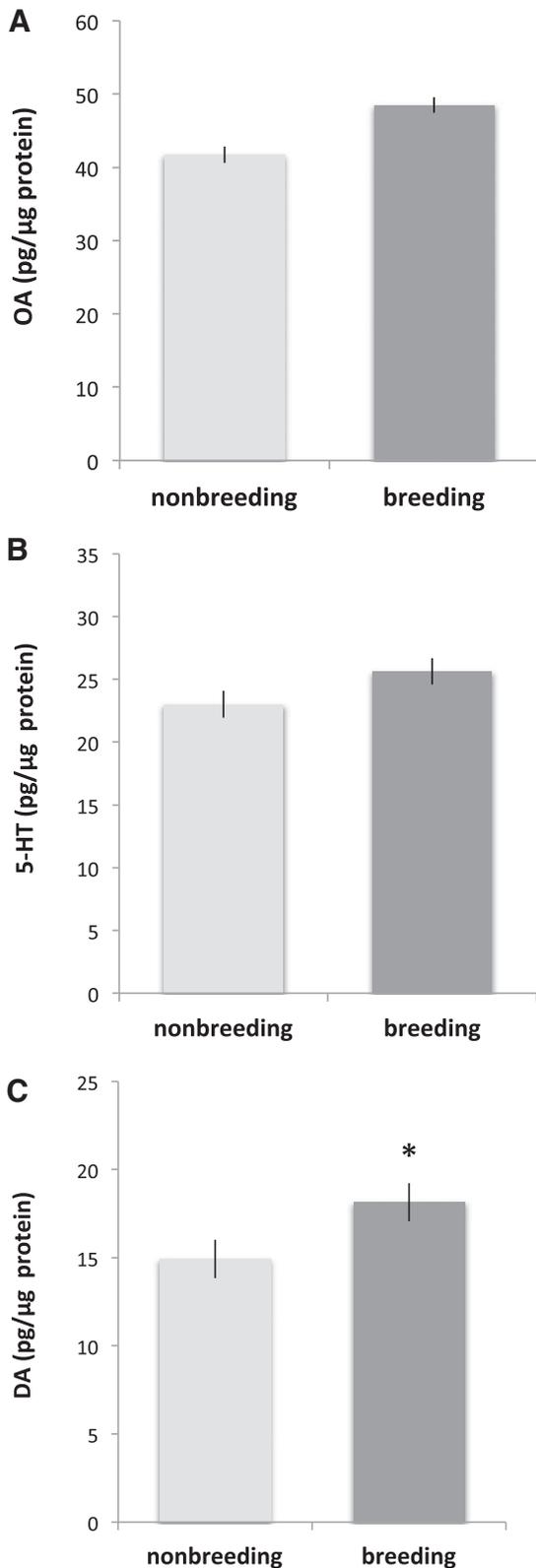
Data were log-transformed for statistical analysis and means and standard errors back transformed for graphical representation. To test for differences in monoamine levels between nonbreeding (control) and breeding (parental) burying beetles, a linear mixed model (SYSTAT 13, San Jose, CA) was used, with breeding status (2 levels) and beetle sex (2 levels) as fixed effects and sampling session as random effect, and applying a critical alpha level of 0.05. *Post hoc* pairwise comparisons were performed in R version 3.2.1 (R Core Team 2015) using the *lsmeans* function and the Tukey method for *P* value adjustment.

## Results

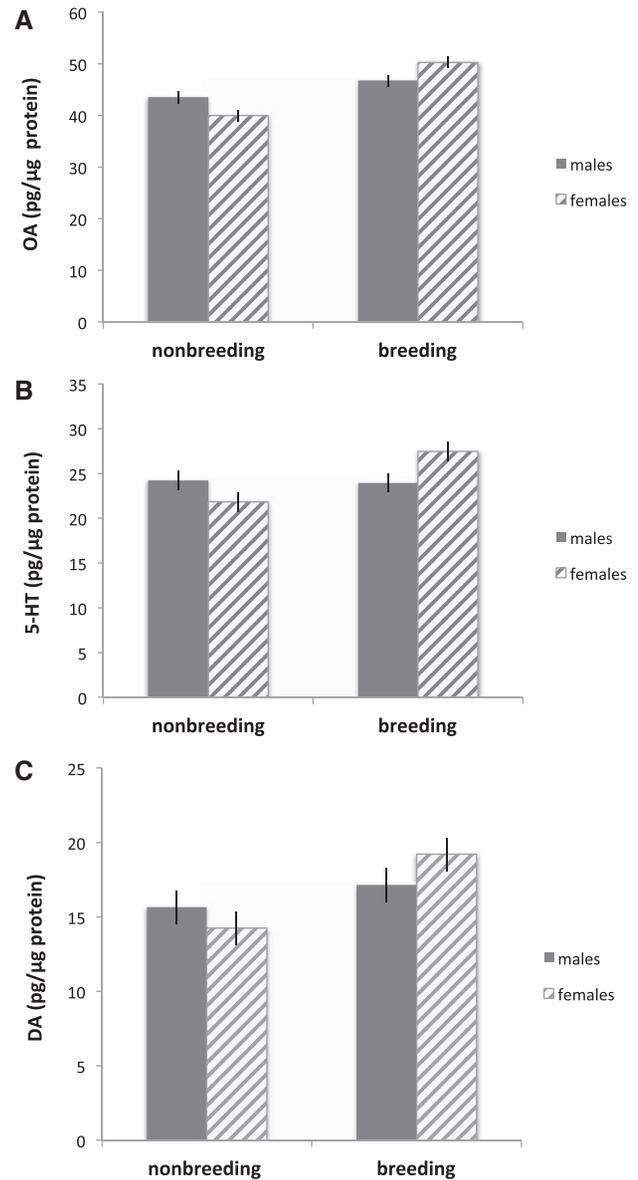
Whole brain levels of each monoamine measured in male and female burying beetles in either nonbreeding status or during the breeding cycle, after 24 h of parental care, are shown in Figure 1A (OA), b (5-HT), and c (DA). OA levels (mean  $\pm$  SE; pg/µg protein) measured in the brains of breeding animals ( $48.5 \pm 1.0$ ) after 24 h of care were not significantly different from levels obtained from nonbreeding pairs ( $41.7 \pm 1.1$ ; Figure 1A). Levels of 5-HT were also similar in breeding and nonbreeding beetles ( $25.6 \pm 1.0$  and  $23.0 \pm 1.0$  pg/µg protein, respectively; Figure 1B). However, dopamine levels increased from  $14.9 \pm 1.0$  pg/µg protein in nonbreeding beetles to  $18.1 \pm 1.0$  pg/µg protein in breeding pairs ( $F_{1,43} = 4.74$ ,  $P = 0.03$ ; Figure 1C). There was no statistically significant effect ( $P > 0.05$ ) of beetle sex on DA levels. Similarly, there was no statistically significant effect of either breeding status or beetle sex, as well no significant interaction of factors ( $P > 0.05$ ) for either OA or 5-HT levels. Central OA levels (pg/µg protein; Figure 2A) measured in both breeding males ( $46.7 \pm 1.1$ ) and breeding females ( $50.2 \pm 1.1$ ) were comparable to those of nonbreeding males ( $43.5 \pm 1.1$ ) and females ( $39.9 \pm 1.1$ ). Brain 5-HT levels (pg/µg protein; Figure 2B) also showed little change between breeding animals ( $23.9 \pm 1.1$  for males and  $27.4 \pm 1.1$  for females) and nonbreeding animals ( $24.2 \pm 1.1$  for males and  $21.8 \pm 1.1$  for females). *Post hoc* pairwise comparisons similarly revealed no statistically significant differences ( $P > 0.05$ ) among brain DA levels between male and female burying beetles in either nonbreeding or breeding status (Figure 2C). Brain DA levels (mean  $\pm$  SE; pg/µg protein) of breeding males ( $17.1 \pm 1.1$ ) and females ( $19.2 \pm 1.1$ ) were similar to those of nonbreeding males ( $15.6 \pm 1.1$ ) and females ( $14.2 \pm 1.1$ ). Of note, we have nevertheless identified a trend toward higher DA levels in breeding (parental) females compared to nonbreeding (control) females ( $P = 0.09$ ).

## Discussion

In the facultatively biparental burying beetles, *N. orbicollis*, reproductive behavior depends on a complex neuroendocrine control that appears to have enabled a vertebrate-like plasticity in the modulation of the onset, intensity and termination of care (Panaitof et al. 2004; Scott and Panaitof 2004). Because this elaborate pattern of



**Figure 1.** Levels (pg/μg protein) of octopamine (OA), serotonin (5-HT), and dopamine (DA) measured in the brains of burying beetles in either nonbreeding status ( $N=24$ ) or after 24 h of parental care ( $N=24$ ). Bars represent mean  $\pm$  SE of OA (A), 5-HT (B), and DA (C) brain levels. A linear mixed model was used to test for differences in brain monoamine levels. Of the three monoamines, only DA showed a significant increase in the brains of parental beetles compared to nonbreeding controls, as denoted by the \* ( $P=0.03$ ).



**Figure 2.** Levels (pg/μg protein) of octopamine (OA), serotonin (5-HT), and dopamine (DA) measured in the brains of male and female burying beetles in either nonbreeding status or after 24 h of parental care ( $N=12$ /beetle sex). Bars represent mean  $\pm$  SE of OA (A), 5-HT (B), and DA (C) brain levels. *Post hoc* pairwise comparisons identified a trend toward higher DA levels in breeding (parental) females compared to nonbreeding (control) females ( $P=0.09$ ).

care depends on coordinated changes in the reproductive behavior and physiology of male and female beetle parents, a thorough investigation of the neuromodulatory and endocrine factors involved is critical for discerning the precise neural and physiological mechanisms enabling such flexible reproductive behaviors. Despite the well-established hormonal correlates of parental behavior, it is not clear how changes in reproductive hormones like JH may be responsible, directly or indirectly, for the organization of reproductive events or for the changes in behavioral state associated with the onset of parental care during a breeding bout. Here, we have begun to explore a potential role for the biogenic amines in the neuromodulation of biparental behavior in the burying beetles.

The significant elevation of DA, but not OA or 5-HT levels, in the brains of parental beetles, compared to unmated controls, is intriguing, especially in the context of exploring a neural mechanism for the transition from the nonbreeding to breeding (parental) behavioral state. During a reproductive bout, the initiation of care, in particular, appears to be a finely tuned behavior: parents will accept and feed larvae that arrive on the carcass when their own are expected to hatch but will cannibalize larvae that hatch either too early or too late (Müller and Eggert 1990; Oldekop et al. 2007). The neurophysiological mechanism underlying this striking change in responsiveness to larvae is not yet understood but it seems likely that it may involve neuromodulation of a behavioral response threshold and subsequent decrease in aggression towards newly hatched offspring.

Our findings thus seem to suggest that DA, but not OA or 5-HT, may be potentially implicated in the onset of care and/or modulation of parental activities in breeding burying beetles. A related question is whether an increase in DA levels may serve to drive the behavioral switch to the parental state, presumably by altering a behavior activation threshold necessary for the initiation of care, or could conceivably arise in response to stimuli from young, facilitating the modulation of parental care activities. Intriguingly, the trend for higher brain DA in parental (breeding) females compared to nonbreeding females, and the lack of a similar trend in breeding males, may similarly reflect a role for DA in mediating the fine adjustments in the intensity of parental care activities between the sexes, and would be in accord with established behavioral sex differences, given that *N. orbicollis* females spend significantly more time provisioning larvae compared to males (Fetherston et al. 1990). The absence of a significant change in either OA or 5-HT brain levels in parental beetles is surprising, given that OA has been implicated in altering the threshold for task-related behavioral transitions (Schulz et al. 2002a, 2002b), while 5-HT linked to the modulation of social context-dependent aggression in a number of insects (Stevenson and Schildberger 2013; Alekseyenko and Kravitz 2014; Bubak et al. 2014a).

A potential role for DA in mediating the transition between conflicting behavioral states, such as the switch from nonparental to parental condition, appears warranted, especially in light of emerging evidence from several recent studies in other social insects. In the highly eusocial honey bees, DA has been implicated in task-related behavioral specialization, with higher brain DA levels in foragers compared to nurse bees (Taylor et al. 1992). Dopamine levels are also elevated in queens but not reproductively suppressed workers (Sasaki et al. 2012b), and experimentally increased DA is associated with ovarian development (Harris and Woodring 1995; Dombroski et al. 2003) and transition to reproductive status in honey bee workers in queenless colonies (Matsuyama et al. 2015). Interestingly, recent studies in the fruit fly, *D. melanogaster*, have implicated DA in motivated behavior and reward signaling processes underscoring learning and appetitive memory formation (Burke et al. 2012; Wadell 2013), while in crickets, DA is necessary to restore aggression and the motivation to fight following defeat in a social contest (Rillich and Stevenson 2014). Taken together, these findings provide some interesting parallels to the well-established role of DA in reward-based, motivated behavior in mammals (Bromberg-Martin et al. 2010). Mammalian neural circuits involved in behavioral activation, as well as regulation of task engagement and effort-related processes, similarly rely on DA, implicating the monoamine in both appetitive and aversive motivational processes (Salamone et al. 2015).

To our knowledge, this is the first study exploring the link between central monoamine levels and breeding status in a biparental insect and our findings seem to indicate that DA may be especially important in orchestrating the behavioral transitions associated with the initiation of care and/or altering a behavioral threshold responsible for the performance of the specialized tasks that it entails. To better understand the role of DA in burying beetle reproductive behavior, we have further set out to explore a potential functional link between JH and DA during breeding, given that both molecules show significant increases associated with the initiation of care. Similarly, to begin to probe the intracellular effects of the three monoamines, we plan to identify and characterize several OA, DA, and 5-HT receptor subtypes in *N. orbicollis*. Related studies will use *in vivo* pharmacological treatments to alter brain monoamine function, either by directly manipulating DA levels via dietary supplementation or inhibition of biosynthesis or by administering specific monoamine receptor antagonists or agonists, followed by assays to uncover any behavioral changes associated with the initiation of care, intensity of parental effort, and duration of care.

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