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RESEARCH ARTICLE

# Neurohormonal changes associated with ritualized combat and the formation of a reproductive hierarchy in the ant *Harpegnathos saltator*

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## ABSTRACT

Dominance rank in animal societies is correlated with changes in both reproductive physiology and behavior. In some social insects, dominance status is used to determine a reproductive division of labor, where a few colony members reproduce while most remain functionally sterile. Changes in reproduction and behavior in this context must be coordinated through crosstalk between the brain and the reproductive system. We investigated a role for biogenic amines in forming this connection in the ant *Harpegnathos saltator*. In this species, workers engage in an elaborate dominance tournament to establish a group of reproductive workers termed gamergates. We analyzed biogenic amine content in the brains of gamergates, inside-workers and foragers under stable colony conditions and found that gamergates had the highest levels of dopamine. Dopamine levels were also positively correlated with increased ovarian activity among gamergates. Next, we experimentally induced workers to compete in a reproductive tournament to determine how dopamine may be involved in the establishment of a new hierarchy. Dopamine levels rose in aggressive workers at the start of a tournament, while workers that were policed by their nestmates (a behavior that inhibits ovarian activity) showed a rapid decline in dopamine. In addition to dopamine, levels of serotonin and tyramine differed among castes, and these changes could contribute to differences in caste-specific behavioral patterns observed among non-reproductive workers. Overall, these results provide support that biogenic amines link changes in behavior and dominance with reproductive activity in *H. saltator* as well as drive differences in worker task performance.

**KEY WORDS: Biogenic amines, Dopamine, Dominance, Aggression, Social insects**

## INTRODUCTION

Social insects have been held as models of cooperation, but closer inspection of their societies has revealed complex dominance orders and high levels of intracolonial aggression in some species (Heinze et al., 1994). Similar to vertebrate societies, social insects may engage in aggressive tournaments to compete over reproductive rights. Dominance position is related to a reproductive division of labor, where only one or a few individuals in a colony reproduce while the rest serve as a functionally sterile workforce. Changes in reproductive status are linked with dominance position, and these reproductive changes may also drive differences in behavior (Amdam et al., 2006; Röseler et al., 1985).

A challenge for understanding the regulation of dominance has been determining the connection between behavioral processing in the brain and changes in the reproductive system. In the social insects, competitions over reproductive rights generally occur among females. Traditionally, changes in ovarian status have been associated with an increase in juvenile hormone (JH) (Hartfelder, 2000; Nijhout, 1994; Raikhel et al., 2005). However, the positive association between JH and reproduction is not universal in social insects. For example, JH levels are lowest in reproductive individuals in some ants and bees (Brent et al., 2006; Penick et al., 2011; Robinson et al., 1991; Robinson et al., 1992; Sommer et al., 1993). This dissociation of JH from reproduction suggests that other factors contribute to the coordinated changes in behavior and physiology associated with reproductive dominance.

In both vertebrates and invertebrates, there is evidence that another class of compounds, the biogenic amines, may regulate behavior associated with dominance hierarchies, including aggression (Kravitz and Huber, 2003; Miczek et al., 2002; Nelson, 2006). In bumble bees, dominant individuals have increased octopamine levels (Bloch et al., 2000c), and a similar pattern has been observed in the 'queenless' ant *Streblognathus peetersi* (Cuvillier-Hot and Lenoir, 2006). Both octopamine and serotonin have been linked to aggressive behavior in crickets (Adamo et al., 1995; Dyakonova et al., 2002; Murakami and Itoh, 2001), and changes in dopamine are associated with dominance in vertebrates (Miczek et al., 2002) and solitary insects (Baier et al., 2002; Stevenson et al., 2005). With respect to reproduction, there is mounting evidence that dopamine stimulates ovarian activity in social insects (Bloch et al., 2000c; Boulay et al., 2001; Dombroski et al., 2003; Kamhi and Traniello, 2013; Sasaki et al., 2007; Sasaki et al., 2009) and may play a role in signaling between the brain and the ovaries (Vergoz et al., 2012).

In addition to reproduction and dominance, biogenic amines affect differences in task performance among non-reproductive workers (Schulz and Robinson, 1999; Seid et al., 2008; Seid and Traniello, 2005; Wagener-Hulme et al., 1999; Wnuk et al., 2011). Workers generally display an age-based polyethism, where young workers perform in-nest duties while older workers transition to outside tasks, such as nest defense and foraging. This transition is accompanied by a well-defined increase in JH (Robinson and Vargo, 1997), but evidence from honey bees suggests that octopamine has a more direct influence on foraging behavior (Schulz et al., 2002). Changes in other amines, such as tyramine and serotonin, also occur in aging workers and influence locomotor activity (Fussnecker et al., 2006) and aggression during nest defense (Kostowski and Tarchalska, 1972; Kostowski et al., 1975).

We investigated the role of biogenic amines with respect to behavior and reproduction in the ant *Harpegnathos saltator* (T. C.

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Jerdon 1851). This species has served as a model for research into the regulation of reproduction (Liebig et al., 1999; Peeters and Hölldobler, 1995; Penick et al., 2011) as well as pheromone signaling of fertility status (Liebig et al., 2000). Colonies of *H. saltator* are founded by a single queen, but after queen senescence, workers compete in a ritualized dominance tournament to decide a new group of reproductives. These reproductive workers, termed gamergates, mate with their brothers (Peeters et al., 2000). Once established, they display dominant behavioral characteristics and serve as the sole egg-layers in the colony. Despite major differences in reproductive potential and behavior, a previous study found no differences in JH or ecdysteroid levels between gamergates and inside-workers (Penick et al., 2011). Workers also do not display changes in the spatial pattern of dopaminergic or serotoninergic activity in their brains during reproductive tournaments (Hoyer et al., 2005). If biogenic amines are involved in dominance relationships in this species, then it is likely that individual differences in the relative amount of specific amines in the brain may drive changes in behavior associated with shifts in reproductive status.

In order to investigate the role of biogenic amines in reproductive regulation in *H. saltator*, we compared brain levels of biogenic amines in gamergates, inside-workers and foragers under stable colony conditions to establish baseline differences. Because the factors that promote the stability of a reproductive hierarchy may differ from the factors involved in establishing a new hierarchy, we measured amine changes associated with the onset of a reproductive tournament. We experimentally induced workers to engage in a dominance tournament and quantified changes in amine levels associated with the onset of aggressive behavior. We also monitored changes after individuals were subjected to policing, an aggressive behavior that strongly inhibits reproduction and reverses dominance status (Liebig et al., 1999). Finally, we measured expression levels of the dopamine receptor genes in the brains and ovaries of workers and gamergates.

## RESULTS

### Caste differences in brain levels of biogenic amines

With respect to reproductive differences, we found that dopamine levels were elevated in gamergates compared with other worker castes (Table 1), while foragers had the lowest levels of dopamine (Friedman's ANOVA, $N$=13, d.f.=2, $\chi^2$=12.92, $P$=0.0016; Wilcoxon signed-rank multiple comparisons, two-tailed: gamergate versus outside-worker, $Z$=–3.11, $P$=0.006; gamergate versus inside-worker, $Z$=–2.34, $P$=0.038; inside-worker versus outside-worker, $Z$=–1.99, $P$=0.046) (Fig. 1A). Serotonin levels were significantly higher in foragers than in both inside-workers and gamergates (Friedman's ANOVA, $N$=13, d.f.=2, $\chi^2$=14.31, $P$=0.0008; Wilcoxon signed-rank multiple comparisons, two-tailed: outside-worker versus inside-worker, $Z$=–3.18, $P$=0.003; outside-worker versus gamergate, $Z$=–2.90, $P$=0.008; inside-worker versus gamergate, $Z$=–1.57, $P$=0.116) (Fig. 1B), while tyramine levels were significantly higher in inside-workers compared with both foragers and gamergates (Friedman's ANOVA, $N$=13, d.f.=2, $\chi^2$=14.92, $P$=0.0006; Wilcoxon signed-rank multiple comparisons, two-tailed: inside-worker versus outside-worker, $Z$=–2.97, $P$=0.009; inside-worker versus gamergate, $Z$=–0.38, $P$=0.009; outside-worker versus gamergate, $Z$=–3.11, $P$=0.701) (Fig. 1C). In contrast to studies on other social insect species, octopamine levels did not differ among groups (Friedman's ANOVA, $N$=13, d.f.=2, $\chi^2$=2.00, $P$=0.37) (Fig. 1D). Because tyramine and octopamine are known to have antagonistic effects on the nervous system (Roeder et al., 2003; Saraswati et al., 2004), we also compared the ratio of octopamine to tyramine between inside-workers and foragers. Tyramine is a precursor of octopamine, but tyramine is also a neuroactive compound in its own right. The ratio of octopamine to tyramine was significantly higher in foragers, indicating that foragers had elevated levels of octopamine compared with tyramine (Wilcoxon signed-rank test, two-tailed, $N$=12, $Z$=–2.82, $P$=0.0048) (Fig. 2).

Dopamine levels in the brain were positively correlated with the number of vitellogenic oocytes in gamergate ovaries (linear regression, $N$=20, $r^2$=0.36, $F$=10.16, $P$=0.0051, best fit: $y$=0.23$x$+0.67) (Fig. 3). Because amine levels were based on three pooled individuals, we counted oocytes from all three gamergates included in each sample and took the average. However, no correlation was found between the number of vitellogenic oocytes and brain concentrations of serotonin, tyramine or octopamine (linear regression; serotonin, $N$=19, $r^2$=0.033, $F$=0.58, $P$=0.46; tyramine, $N$=19, $r^2$=0.0062, $F$=0.11, $P$=0.75; octopamine, $N$=20, $r^2$=0.018, $F$=0.33, $P$=0.57).

### Biogenic amine levels and dominance behavior

Workers had significantly higher levels of dopamine 3 days after the start of dueling, while levels of other biogenic amines were unchanged (Wilcoxon signed-rank test, two-tailed: dopamine, $N$=21, $Z$=–2.03, $P$=0.042; serotonin, $N$=21, $Z$=–0.86, $P$=0.39; tyramine, $N$=21, $Z$=–0.54, $P$=0.59; octopamine, $N$=21, $Z$=–0.96, $P$=0.339) (Fig. 4A). In response to policing, brain levels of dopamine, serotonin and tyramine were significantly reduced in workers that received policing, while octopamine levels did not change (Wilcoxon signed-rank test, two-tailed: dopamine, $N$=15, $Z$=–2.73, $P$=0.0064; serotonin, $N$=15, $Z$=–2.95, $P$=0.0031; tyramine, $N$=15, $Z$=–2.33, $P$=0.020; octopamine, $N$=14, $Z$=–0.41, $P$=0.68) (Fig. 4B).

### Expression of dopamine receptor genes in brains and ovaries

*Harpegnathos saltator* has three dopamine receptor genes (*Hsal-dop1*, *Hsal-dop2* and *Hsal-dop3*), which are orthologs of dopamine receptor genes found in honey bees and *Drosophila melanogaster* (Bonasio et al., 2010) and may have similarities to dopamine receptor genes in vertebrate species (Mustard et al., 2012). All three dopamine receptor genes were expressed in brains of gamergates

**Table 1. Worker caste characteristics of *H. saltator* and relative biogenic amine levels**

| | | | | Relative biogenic amine levels | | |
|---|---|---|---|---|---|---|
| | Age (days) | No. yolky oocytes | Task performance | DA | 5-HT | TA |
| Gamergate | 50–900 | 4–9 | Reproduction | High | Low | Low |
| Inside-worker | 0–50 | 0–2 | Brood care | Medium | Low | High |
| Forager | 50–200 | 0 | Nest defense, foraging | Low | High | Low |

DA, dopamine; 5-HT, serotonin; TA, tyramine.
Levels of octopamine did not differ among groups and are therefore not included.
Age values are estimates based on personal observations as well as previous data (Haight, 2012; Peeters et al., 2000).

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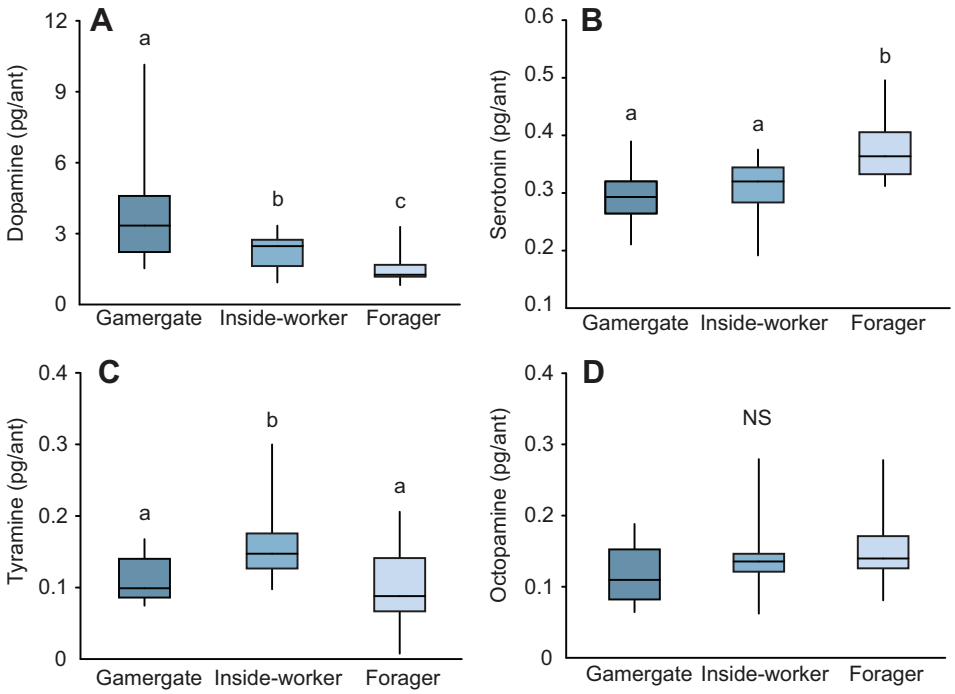


**Fig. 1. Caste-based differences in biogenic amine levels.** Median, 25–75% and range of brain levels of (A) dopamine, (B) serotonin, (C) tyramine and (D) octopamine in gamergates, inside-workers and foragers during stable colony conditions. Different lowercase letters indicate significant differences ($P$<0.05, Wilcoxon signed-rank, $N$=13 for each caste).

and workers ($N$=10 colonies), but only the receptor genes for *Hsal-dop1* and *Hsal-dop3* were consistently expressed in the ovaries at quantifiable levels (Fig. 5). The level of expression in the brain was 2–3 orders of magnitude higher than the expression of these receptor genes in the ovaries (expression ratio from brain to ovaries: *Hsal-dop1*, 25:1; *Hsal-dop3*, 120:3; note, only fold-change is reported in Fig. 5, so these differences are not apparent). With respect to differences of dopamine receptor expression in the brain, gamergates showed decreased levels of *Hsal-dop1* (Wilcoxon signed-rank test, one-tailed, $N$=10, $Z$=–1.78, $P$=0.038), while no differences were observed in the relative expression of *Hsal-dop2* or *Hsal-dop3* (Wilcoxon signed-rank test, one-tailed, $N$=10, *Hsal-dop2*: $Z$=–17.03, $P$=0.36; *Hsal-dop3*: $Z$=–1.38, $P$=0.084) (Fig. 5A). In ovarian tissue, the expression levels of *Hsal-dop1* and *Hsal-dop3* were decreased in gamergates compared with workers (Wilcoxon signed-rank test, one-tailed, $N$=10, *Hsal-dop1*: $Z$=–1.78, $P$=0.038; *Hsal-dop3*: $Z$=–2.19, $P$=0.014) (Fig. 5B).

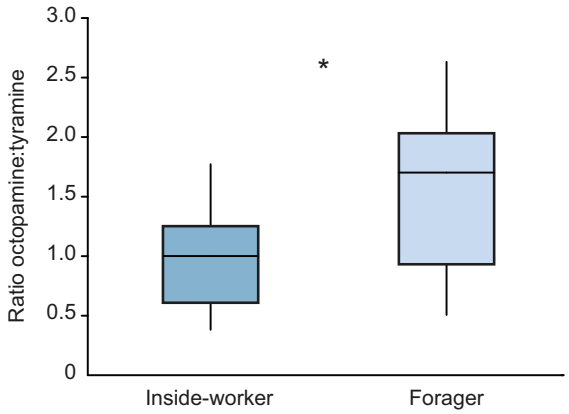


**Fig. 2. Ratio of octopamine to tyramine in workers.** Median, 25–75% and range of the ratio of octopamine to tyramine in individual inside-workers and foragers (*$P$=0.0029, Wilcoxon signed-rank, $N$=12).

## DISCUSSION

Differences in dopamine levels among castes clearly corresponded with a reproductive division of labor in *H. saltator*. Gamergates had the highest levels of dopamine, and dopamine levels were positively correlated with the number of yolky oocytes in gamergate ovaries. In other social insects, JH and ecdysteroid levels have been found to differ with respect to reproductive dominance (Bloch et al., 2000a; Bloch et al., 2000b; Brent et al., 2006; Giray et al., 2005; Sommer et al., 1993), but levels of these hormones do not differ between gamergates and workers in *H. saltator* (Penick et al., 2011). JH is thought to stimulate uptake of vitellogenin (yolk protein) by the ovaries in solitary insects, but JH has apparently lost this function in adult reproductives of honey bees (Robinson and Vargo, 1997) and some ants (Brent et al., 2006; Sommer et al., 1993). Instead, there is

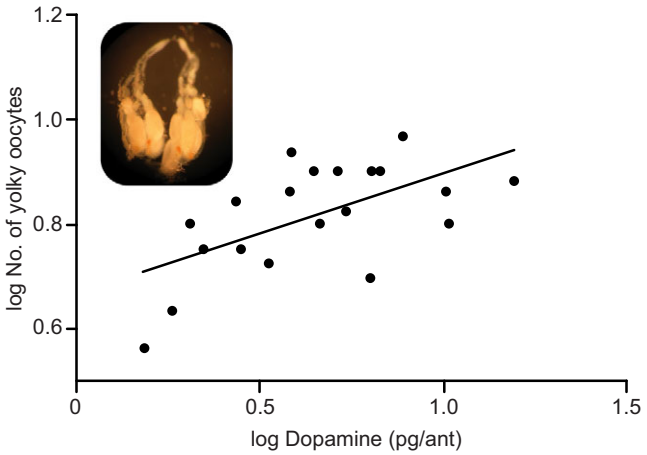


**Fig. 3. Brain dopamine levels and number of yolky oocytes in gamergate ovaries.** Levels of dopamine were positively correlated with the number of yolky oocytes in gamergate ovaries, a measure of reproductive status (linear regression, $N$=20, $r^2$=0.36, $P$=0.0051; line: $y$=0.23$x$+0.67). Inset shows example of gamergate ovaries with yolky oocytes visible.

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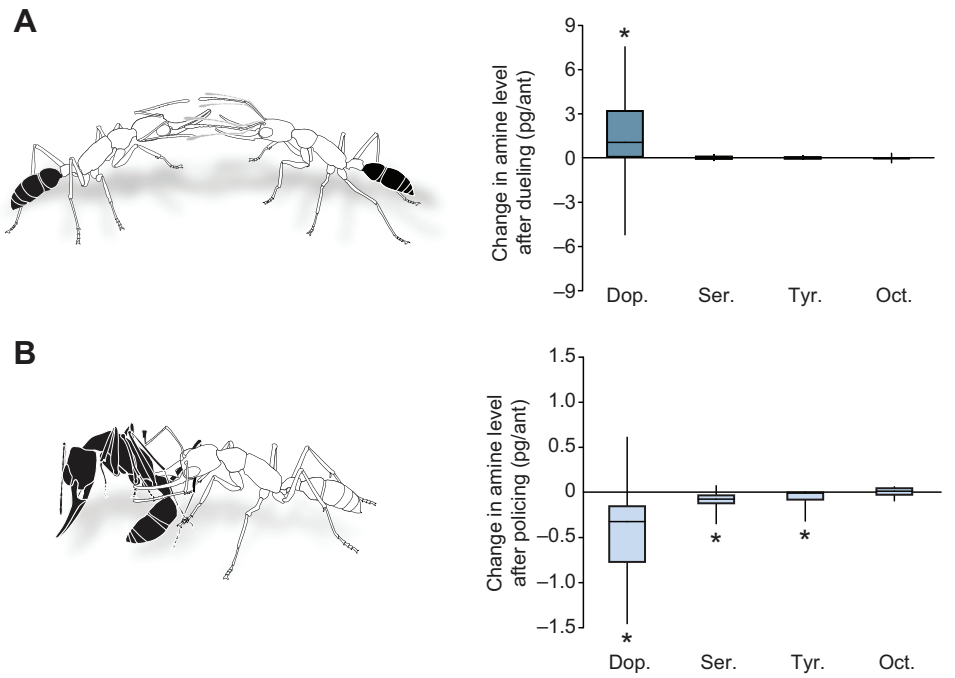


**Fig. 4. Changes in biogenic amines during reproductive tournaments.** (Right) Median, 25–75% and range of the change in biogenic amines (A) 3 days after the onset of dueling (*N*=21) and (B) 24 h after policing (dopamine, serotonin, tyramine: *N*=15; octopamine: *N*=14). Asterisks indicate values significantly different from zero ($P<0.05$). Diagrams of duelling and policing are shown on the left.

increasing evidence in *Apis mellifera* that dopamine serves as a gonadotropin (Brandes et al., 1990; Harris and Woodring, 1995; Mustard et al., 2012; Vergoz et al., 2012), and our results suggest this could be true for other social insects, including ants.

Dominance in some social insect species has been associated with elevated octopamine (Bloch et al., 2000c; Cuvillier-Hot and Lenoir, 2006), but octopamine levels in gamergates of *H. saltator* did not differ from those of non-reproductive workers. Because octopamine has been directly linked to aggressive behavior in *Drosophila* males (Hoyer et al., 2008) and fighting crickets (Adamo et al., 1995), we hypothesized that octopamine may play a role during reproductive tournaments, when workers display the highest frequency of aggressive behavior. When we experimentally induced dominance tournaments in *H. saltator*, we did not see a change in octopamine levels. Instead, we saw an increase in dopamine at the onset of dueling, which suggests that dopamine plays a role in both the establishment and the maintenance of dominance in *H. saltator*.

With respect to behavior, increased levels of dopamine have been previously associated with dominance and aggression in vertebrates (Miczek et al., 2002). In *H. saltator*, dopamine levels increase at the onset of a reproductive tournament, but after the tournament is over, dopamine levels remain high in stable gamergates. Stable gamergates do not often engage in aggression, so dopamine is probably not directly associated with aggressive behavior. Instead, dopamine may serve as a neuromodulator, whereby individuals with elevated dopamine may be more likely to respond to aggression with a dominant response rather than a subordinate response. Gamergates consistently display a dominant posture (tall stance with an elevated gaster), and when they are confronted by a challenging worker they often respond with dominance biting. But based on our results, it is not clear what factors are directly driving aggressive displays during reproductive tournaments.

In response to worker policing, dopamine levels declined, which fits the prediction that dopamine levels coordinate dominance and reproductive activity in *H. saltator*. Worker policing is a behavioral mechanism that inhibits ovarian activity in the individual that is policed. The drop in dopamine 24 h after policing is likely part of a cascade of events that leads to a decrease in ovarian activity and resorption of developing oocytes. We also found a decrease in serotonin and tyramine levels, but these neurohormones are already low in gamergates compared with other castes, and levels of these amines did not change during the onset of reproductive tournaments.

Workers may be targeted for policing based on the display of a chemical fertility signal (Smith et al., 2009), and dominance status in stable colonies of *H. saltator* is presumably maintained through the production of a distinct cuticular hydrocarbon profile (Liebig et al., 2000). In order for this signal to be a reliable indicator of ovarian activity, it must be linked to a clear indicator of ovarian status. While

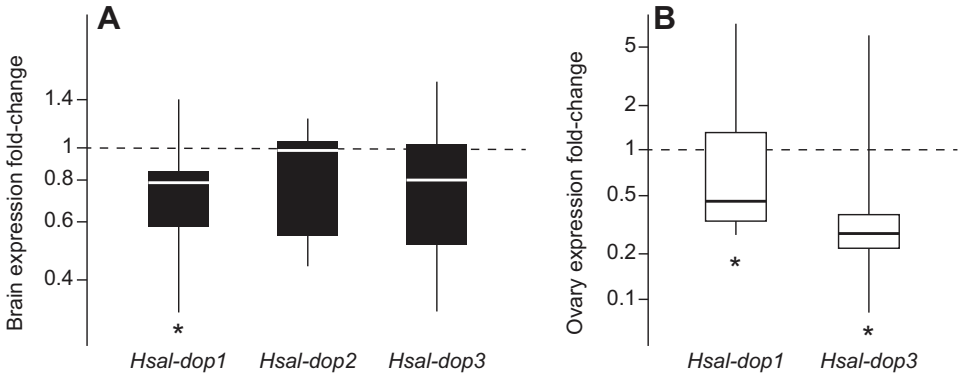


**Fig. 5. Fold-change in dopamine receptor expression in gamergates compared with workers.** Median, 25–75% and range of expression fold-change (log scale) in (A) brains and (B) ovaries standardized to expression levels of GAPDH (*N*=10). Note: *Hsal-dop2* was not expressed at measurable levels in the ovaries of either gamergates or workers. Asterisks indicate a significant difference in expression level in gamergates compared with workers ($P<0.05$, Wilcoxon signed-rank, *N*=10).

JH levels have been linked with the production of a fertility signal in another ant species, *Streblognathus peetersi* (Cuvillier-Hot et al., 2004), dopamine is a more likely candidate in *H. saltator*. Dopamine has been connected to the production of a female-specific hydrocarbon pattern in *Drosophila* (Marican et al., 2004), and dopamine and its receptors are found in insect cuticle (Evans, 1981). After policing, dopamine levels decrease in a short time span, and this may force policed workers to shut down their ovaries and the production of the fertility signal. Only after this is accomplished are they able to reintegrate into the colony as a non-reproductive worker.

### Worker division of labor

In addition to a reproductive division of labor, non-reproductive workers of *H. saltator* display a temporal polyethism, where young workers perform in-nest tasks while older workers transition to foraging (Haight, 2012). A long history of research has focused on a connection between increased JH levels and foraging in social insects (Dolezal et al., 2012; Hartfelder, 2000), and we previously confirmed this pattern in *H. saltator* (Penick et al., 2011). Alternatively, work on biogenic amines in honey bees has found that octopamine may regulate foraging in tandem with JH, and the response to octopamine treatment is more rapid than the response to JH treatment (Schulz et al., 2002). In spite of this, we did not find differences in octopamine levels among castes of *H. saltator*. Instead, we found the ratio of tyramine to octopamine to be higher in inside-workers than in foragers. Tyramine is the precursor of octopamine, and the ratio of tyramine to octopamine may be important for regulating behavior (Roeder et al., 2003; Saraswati et al., 2004). Tyramine is also a neuroactive compound in its own right with its own receptors. In honey bees, tyramine has been shown to modulate locomotor activity, and injection of tyramine into worker bees caused a reduction in flying, a behavior associated with foraging (Fussnecker et al., 2006). Similarly, increased tyramine in inside-workers of *H. saltator* may inhibit foraging.

Foragers were distinguished by having elevated serotonin levels compared with both inside-workers and gamergates. Serotonin has been shown to affect circadian cycles in numerous insect species (Page, 1987; Tomioka et al., 1993; Yuan et al., 2005) and could affect foraging cycles. Foraging in *H. saltator* is related to daily light cycles, but gamergates and inside-workers are usually not exposed to daylight in the wild because they remain inside the nest. We controlled for time of day when we collected foragers for this study (foragers were collected in mid-afternoon, during the 'daylight' period in our rearing facility), so it is possible that serotonin levels increase in daylight hours and decrease during lower levels of foraging activity.

Alternatively, serotonin has been associated with aggression in other arthropods (Kravitz and Huber, 2003) and has been linked with defensive behavior in honey bees (Hunt, 2007) and ants (Kostowski et al., 1975; Seid et al., 2008). We did not find a connection between serotonin and aggressive behavior during reproductive tournaments in *H. saltator*, but the effects of serotonin could be specifically related to aggression in a defensive context. Gamergates and inside-workers are timid towards foreign intruders in *H. saltator*, but foragers exhibit defensive displays and actively attack intruders when provoked. The studies that have linked serotonin with aggression in social insects have also focused on the defensive context, so it is possible that serotonin may serve this role in *H. saltator* as well.

Because task performance in *H. saltator* is correlated with age, we cannot rule out the possibility that the patterns we observed are associated with behavioral maturation rather than task performance. For example, in the wood ant *Formica polyctena*, octopamine levels are lower in foragers than in younger nest workers, but when foragers are reverted back to nest-worker status, their octopamine levels remain low (Wnuk et al., 2011). Therefore, changes in octopamine levels in this case are related to behavioral maturation of workers rather than task performance per se. It is important to note, however, that in the present study gamergates and foragers are comparable in age and are both older than inside-workers. It is interesting, then, that gamergates and foragers show opposite trends with respect to dopamine and serotonin levels, and only with respect to tyramine do they display a pattern that is correlated with age.

### Changes in brain and ovary tissue

Our results point to a connection between brain levels of dopamine and ovarian activity in *H. saltator*, but it is unclear specifically how dopamine affects the ovaries. A recent study in honey bees found dopamine receptors in both the brain and ovaries, with expression levels that varied by reproductive status (Vergoz et al., 2012). We found expression of three dopamine receptor subtypes (*Hsal-dop1*, *Hsal-dop2* and *Hsal-dop3*) in the brain of *H. saltator* and two subtypes (*Hsal-dop1* and *Hsal-dop3*) in the ovaries. This pattern is similar to what was recently reported in *A. mellifera*, where all three receptor subtypes are expressed in the brain, but only orthologs of *Hsal-dop1* and *Hsal-dop3* (*Amdop-1* and *Amdop-3*, respectively) are expressed in the ovaries (Vergoz et al., 2012). Therefore, it is possible that these receptors have a generalized function in the ovaries that may potentially relate to a gonadotropic effect of dopamine. However, expression levels for dopamine receptor genes were several orders of magnitude lower in the ovaries than in the brain. Also, gamergates showed an overall decrease in dopamine receptor expression in the brain and ovaries compared with workers. While it is not clear whether lower expression levels correlate with a decrease in dopamine sensitivity, it does raise questions about how differences in dopamine content and receptor expression modulate an individual's response. Additional work on the molecular action of dopamine will be necessary to tease these actions apart.

As workers of *H. saltator* become reproductive and shift to gamergate status, they exhibit a reduction in brain volume with an especially strong reduction of the optic lobes (Gronenberg and Liebig, 1999). Studies in humans (Xu et al., 2002) as well as *Drosophila* (Bayersdorfer et al., 2010) have found a link between high dopamine levels and neurodegeneration, and this connection may play an important role in the negative effects of Parkinson's disease. The correlation between high dopamine levels and a decrease in brain volume in *H. saltator* is intriguing and suggests that dopamine may play a role in brain plasticity in this species. In *H. saltator*, the decrease in brain volume is thought to be an adaptive response, where reproductive individuals reallocate resources from maintaining their central nervous system to fuel their dramatically increased reproductive output. This change also coincides with an increase in gamergate lifespan, where gamergates can live up to 3 years (Peeters et al., 2000) while worker lifespan is generally less than 1 year (Haight, 2012). In future studies it would be interesting to explore further the connections between increased dopamine levels and gamergate traits, such as decreased brain volume and increased lifespan.

## MATERIALS AND METHODS

### Study species and laboratory conditions

Whole colonies of *H. saltator* were originally collected in southwestern India as described elsewhere (Peeters et al., 2000). Over 250 stock colonies

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of *H. saltator* were maintained in the laboratory at a constant temperature of 25°C on a 12 h:12 h light:dark cycle. Colonies were fed biweekly with live crickets (*Acheta domesticus*) and housed in plastic boxes (19×27 cm) with a dental plaster floor that featured a preformed nest cavity covered by a glass plate (12×15 cm). Only mature colonies were used for this study (150–350 workers).

### Biogenic amine profiles of worker castes

In order to establish caste-specific biogenic amine levels, workers were divided from 13 colonies into three behavioral groups: gamergates, inside-workers and foragers. These castes differ with respect to age, reproductive status and task performance (Table 1). Methods for distinguishing castes in *H. saltator* have been previously described (Penick et al., 2011). Gamergates were identified based on direct observation of egg-laying and/or the display of dominant behavior. The reproductive status of gamergates was later confirmed by dissection, and the number of yolky oocytes in each individual was quantified as a measure of ovarian activity. For this study, we selected colonies with a stable reproductive hierarchy, and colonies with a high level of intra-colonial aggression were excluded. In all cases, gamergates came from colonies that contained multiple reproductives that were identified at least 6 months prior to sampling to ensure they were mature. Foragers were taken directly from the foraging arena, and individuals were only selected as foragers if they responded with a defensive display when provoked with forceps (Penick et al., 2011). To select inside-workers, all workers present in the foraging arena were first removed, and the colony was allowed to sit for 1 h. After this period, all subsequent workers that entered the foraging arena were also removed, and inside-workers were selected from among the pool of workers that remained inside the nest. Further care was taken to select workers that had been observed actively tending larvae and those workers that had a light-colored cuticle (young workers generally perform nest duties and are lighter in color than older foragers). Fully callow workers with a light cuticle (newly emerged) were excluded from this study.

### Experimental induction of dominance behavior

To examine changes in biogenic amine levels associated with the formation of a new dominance order, we experimentally induced workers to begin a dominance tournament in 21 separate colonies. We induced the performance of two specific behaviors: (1) dueling, which is associated with the establishment of new reproductives, and (2) policing, which is associated with the strong inhibition of reproduction. Sixty to eighty workers in each colony were paint-marked with an individual code using Testors Pactra enamel (Rockford, IL, USA). All mature gamergates were identified and removed to induce workers to begin a dominance tournament, and three randomly selected inside-workers were removed at the same time to serve as a base-level control for dueling worker samples. Three days after tournaments began, we observed colonies to identify workers that were dueling. Workers in each colony that were consistently observed dueling during three, 10 min observation sessions separated by at least 1 h were collected as dueling worker samples (three workers pooled per sample). In order to collect policed workers, we allowed tournaments to continue for 18 days, and we then identified six workers in each colony that displayed dominant behavior (consistent dueling and gamergate-like characteristics). Of these workers, three were sampled immediately to serve as a base-level control for comparison with workers that were policed (grabbed and held by their nestmates), and three additional workers were placed into a satellite nest that contained mature gamergates to induce policing. Focal colonies and satellite nests were derived from the same parent colony. We observed satellite nests at 1 h intervals until policing was observed. Policed workers were collected for amine quantification 24 h after being introduced into satellite nests.

### Biogenic amine quantification

Individual ants were collected from each colony directly into liquid nitrogen, and brain dissections were performed within 3 h of collection. In all cases, each sample included brains pooled from three workers taken from the same colony, and sample size reflects the number of independent colonies that workers were taken from for amine analysis. Amine levels are reported as pg/ant by dividing the total quantity of each amine by the number of workers included in each sample. Pooling brain samples within colonies was necessary to get measurable levels of biogenic amines but it does decrease resolution with respect to individual brain amine levels. The central brain was dissected from the head (without optic lobes) and stored at −80°C until analysis. Three brains per sample were pooled from individuals from the same colony to amplify quantification. After brains were removed, the ovaries were dissected to confirm reproductive status, and the vitellogenic oocytes were counted in gamergates to quantify their level of ovarian activity (Liebig et al., 2000).

For biogenic amine analysis, brains were placed in a 1.5 ml centrifuge tube and homogenized with 20 µl of chilled perchloric acid (0.2 mol $l^{-1}$) that contained dihydroxybenzylamine (DHBA, 87 pg $\mu l^{-1}$) and synephrine (50 pg $\mu l^{-1}$) as internal standards. The samples were sonicated in an ultrasonic bath filled with an ice-water slurry for 5 min, chilled an additional 20 min, and then centrifuged at 12,000 ***g*** for 10 min at 4°C. The biogenic amine content of 10 µl of the supernatant was analyzed by high-pressure liquid chromatography (HPLC). The HPLC system (ESA, Chelmsford, MA, USA) consisted of a Coularray model 5600A with a 4-channel electrochemical detector, a model 582 pump and a reverse-phase catecholamine HR-80 column. Samples were delivered via a manual injector (Rheodyne 9125) with a 20 µl loop. Channel 1 was set at 650 mV for octopamine and tyramine. Channel 2 was set at 425 mV for dopamine and serotonin. Amine identity was confirmed by peak responses on a third channel set at 175 mV. The mobile phase was composed of 15% methanol, 15% acetonitrile, 1.5 mmol $l^{-1}$ sodium dodecyl sulfate, 85 mmol $l^{-1}$ sodium phosphate monobasic, 5 mmol $l^{-1}$ sodium citrate and polished water (Barnstead Nanopure). The pH was adjusted to 5.6 using phosphoric acid. The flow rate of the mobile phase was 1 ml $min^{-1}$. Per brain concentrations of the amines were calculated from the peak areas using titer curves of external standards run prior to the samples and after every 10 injections.

### qRT-PCR of dopamine receptor gene expression in brain and ovary tissue

To investigate the possibility of cross-talk between the brain and the ovaries, we measured the expression of dopamine receptor genes (*Hsal-dop1*, *Hsal-dop2*, *Hsal-dop3*) in brain and ovarian tissue of gamergates and workers. These receptor genes were previously identified as orthologs to the dopamine receptor genes found in *A. mellifera* and *D. melanogaster* (Bonasio et al., 2010). In order to reconfirm these gene annotations, we did a BLAST search through NCBI of protein sequences for dopamine receptor genes in *A. mellifera* (*Amdop1*, *Amdop2* and *Amdop3*) against the *H. saltator* genome (see supplementary material Tables S1–S3 for primers, coding sequences and protein sequences used). Gamergates and workers were taken from 10 separate colonies, and brain samples for each caste were pooled from two individuals from each colony. For ovarian tissue, gamergate samples contained ovaries from two individuals while worker samples contained ovaries pooled from five individuals to compensate for their smaller size (Peeters et al., 2000). Expression levels for dopamine receptor genes were standardized based on expression levels of the housekeeping gene GAPDH because GAPDH was found to have a more consistent expression pattern among castes than other common standards, such as actin (C.A.P., C.S.B., K.D. and J.L., unpublished data).

Immediately after dissection, tissues were frozen in pre-chilled tubes on dry ice. Samples were stored at −80°C until RNA extraction. Total RNA was isolated from dissected tissues using Trizol reagent (Life Technologies, Foster City, CA, USA) according to manufacturer's specifications, and RNA was treated with TurboDNase (Ambion, Life Technologies) to remove DNA contamination. RNA integrity and concentration were determined with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) platform. One-step qRT-PCR was performed in triplicate using the ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA). Primers were used at a concentration of 0.6 µmol $l^{-1}$ to amplify 2 ng RNA template in a 20 µl reaction volume. Negative control (without reverse transcriptase) and melting curve analyses confirmed that the qRT-PCR analysis was not confounded by DNA contamination or primer dimers. Dopamine gene expression was normalized to GAPDH levels using a modification of the delta–delta CT method (Pfaffl et al., 2002). Briefly, the

average Ct of the three replicates for each reaction ($x$) was used to calculate relative concentration ($2^{-x}$). Percentage relative concentration of GAPDH was then calculated for *Hsal-dop1*, *Hsal-dop2* and *Hsal-dop3* in each tissue.

## Statistical analyses

All statistical comparisons were analyzed using Statistica version 7 (StatSoft, Tulsa, OK, USA) with alpha set to 0.05. Friedman's ANOVA tests were used to compare differences in biogenic amine levels among castes, and the Wilcoxon signed-rank test was used for multiple comparisons with $P$-values adjusted for a sequential-Bonferroni correction. For all other between-group comparisons that did not involve multiple comparisons, we used Wilcoxon signed-rank tests without adjusted $P$-values. The relationships between amine levels and ovarian development in gamergates were tested using linear regression. We included gamergates from seven additional colonies in this study (20 colonies total), and with this larger sample size all variables conformed to the assumption of normality required for regression analyses. Differences in expression of dopamine receptor genes between gamergates and workers were reported as fold change, but statistical comparisons between castes were based on a one-tailed Wilcoxon signed-rank test using the actual expression values standardized to GAPDH rather than fold-change.

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

C.A.P., J.L. and C.S.B. conceived of the study and drafted the manuscript. C.A.P. designed and carried out all experiments and data analyses. K.D. developed and provided assistance for gene expression studies and qRT-PCR analyses with C.A.P. All authors read and approved the final manuscript.

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### Supplementary material

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**Table S1.** Dopamine receptor primers for *H. saltator*

| Receptor | Forward | Reverse |
|---|---|---|
| *Hsal-dop1* | TGACGAAGCTGCCGGAC | TGGTCGGACACGTGGTAAGG |
| *Hsal-dop2* | AATCTCTGGGAACTCGCGAC | CAAAGTAATTGGTCGCTGTGTG |
| *Hsal-dop3* | ATAGAAGAGTATGGCTGACG | GACAGGCTCGAGTAGATG |
| *Hsal-gapdh* | TATCAAGGCCAAGGTAAAGGA | CATACCACGAGATCAGCTTCA |

**Table S2.** Dopamine receptor coding sequences (primer regions highlighted)

| | |
|---|---|
| ***Hsal*Dop-1** | ATGACCTTCGCCGGGGTCAACGACCTCCTCGGCTATTGGGTCTTCGGCTT<br>GTGGTTCTGCGACATCTGGATCGCCTTCGATGTTATGTGCAGCACTGCCT<br>CTATCCTCAATCTATGCGCCATCTCCCTCGACCGTTACATTCACATTAAG<br>GATCCCCTCAGGTACGGCCGTTGGGTGACTAGAAGAGTCGCTATCGGCGG<br>TATCGCCGTCGTGTGGCTTTTAGCAGGACTCATATCCTTCGTACCGATCA<br>GCCTAGATCTTCACAGGGCAGATCAACCGGCGCTCTATAATGACGGAATA<br>GAGGAGCACCCTACGTGTGCCCTAGACATTACACCCACTTACGCGGTGGT<br>GTCCTCTTGCATATCTTTCTACGTGCCCTGCATCGTGATGTTGGGGATTT<br>ATTGCAGGCTCTATTGCTACGCGCAGAAACACGTAAGGAGTATCCGAGCG<br>GTGACGAAGCTGCCGGACACCTCCATGGCCAAGAGTTTTCGCTCCAAGAG<br>CAGTCGCTGTAAGCCACCGAAGCCGCAAACGAAGACGAAGCCGACTAGTC<br>CTTACCACGTGTCCGACCACAAGGCCGCGATCACTGTCGGCGTAATCATG<br>GGTGTGTTCCTGATATGTTGGGTACCCTTCTTCTGCGTCAACATTGTCGC<br>AGCCTACTGCAAAACCTGCATACCTCTCCGAGCGTTCCAGGTTCTCACGT<br>GGCTCGGCTACAGTAACTCGGCCTTCAACCCGATAATCTACAGCATCTTC<br>AACACTGAGTTCCGGGAGGCGTTCAAGAGGATCCTCACGAAAGGAGCGCG<br>CGCGAGGGGTAATCAGCCGTCGACCAGCGAATGCGGCGAGTTCCGCTCGG<br>TGGTGGTGCAGAAACGCAACGGCTCCATGGTCGAGTGCAACATCAGTCCA<br>AGGTCGAGCGCGGACAGCTGCCAGGTCGGCGTCATGGCTCAAAGGCATCG<br>CGACACTATCGTCAGCGCCATATAA |
| ***Hsal*Dop-2** | ATGAACGACAGCGAGATCTATTTACTGAGCTGGGAAGACGAGGTACACGC<br>AACAAACAACGACGATCTCGGCAGGAGCTTTTACAACGCAAGCTATCCGC<br>CGTTCAACAGCAGCTACGAGAATCTCTGGGAACTCGCGACTGATCGCGCC<br>GGACTCGCGATCGTTCTCCTGCTCTTCTCCGTAGCGACCGTCTTCGGCAA<br>CACGCTGGTGATATTGGCGGTGTTCAGGGAACGGTACCTGCACACAGCGA<br>CCAATTACTTTGTAACCTCATTGGCCTTCGCCGATTGCCTGGTCGGCCTG<br>GTGGTGATGCCGTTCAGCGCGGTTTACGAGGTGCTGGAGAACCGCTGGCT<br>CTTCACGACCGACTGGTGCGACGTGTGGCGCTCGTTGGACGTATTATTCT<br>CCACCGCGTCCATCCTGAATCTGTGCGTCATCAGTTTGGACCGTTACTGG<br>GCGATCACCGATCCGTTCACGTATCCGACGCGGATGAGCCGGAAACGCGC<br>GGCCATCCTCATCGCGATCGTGTGGATCTGCTCGAGCGCGATCTCCTTCC<br>CGGCGATCGCCTGGTGGCGGGCGGTGCGGACCGAACAAGTGCCCGAGGAC<br>AAGTGCCCGTTCACGGAGAACCTCGGCTACCTCATCTTCTCGTCGACAAT<br>CAGCTTCTACCTGCCGCTCTTCGTCATGGTGTTCACGTATTACCGAATCT<br>ATCGCGCCGCCGTGATACAGACGAGGAGCCTGAAGCTCGGTACGAAGCAA<br>GTGATGATGGCCTCGGGCGAGCTCGAGCTCACTCTGAGGATACACCGGGG<br>TGGTGGTACCAACACCGACGCCCGCCACCTCTTTCGAACCACTTCGAGCA<br>CGCCCGAGGAGCTGCAGGATCTCGAGGAGCCGCTAACCGCGCTTCACAAC<br>AACGGTCTCACCCGGGTGCCGTCCGCGAGGCACACCATCAACAACAAGCA<br>ACACCTCGGTAAAAACTTCTCCTTGTCGCGCAAGCTCGCCAAATTCGCCA<br>AGGAGAAGAAGGCGGCCAAGACTTTGGGCATCGTCATGGGCGTCTTCATC<br>ATCTGCTGGCTGCCGTTCTTCGTCGTGAATCTATGGTCGGGATTCTGCAC |

| | |
|---|---|
| | GAGGTGCATCTGGCAGGAAGAGATAGTATCCGCGGCCGTCACATGGCTCG<br>GTTGGATTAATAGTGGAATGAATCCCGTGATATACGCTTGCTGGAGCAGG<br>GACTTCCGTAGGTGA |
| ***Hsal*Dop-3** | GTGTTTCTGCAGGTGAACGGATCTTGGAGTCTGCCTGGATTTGTTTGTGA<br>CTTTTACATCGCAATGGATGTGACTTGCAGTACCAGCTCCATATTCAACC<br>TCGTGGCTATTTCCATAGACAGATACATAGCGGTGACCCAGCCGATAAAG<br>TACGCGAAGCACAAGAACAATAGAAGAGTATGGCTGACGATACTGTTGGT<br>CTGGGCGATATCGGCCGCGATCGGCAGCCCGATTGTCCTAGGCTTGAATA<br>ACACCCCCGACCGGATACCGGACCAATGTCTGTTCTACAATACGGATTTT<br>ATCATCTACTCGAGCCTGTCCAGCTTCTACATACCCTGCATCATCATGGT<br>ATTCCTCTATTATAATATATTCAAGGCTCTGCGAAATAGAGCGAGAAGGG<br>CTCGTGCTAGCAAAAAACCGAATTTAGGCGATATAAAACCGGGAAGCATC<br>ATCGAGAACATCGCACACACGCGCAGGTTTGCGGAAACGGCGTTGGGGGC<br>GGCCGCCTTAGTGGCTCCTGGAATCGAGGAACCGACAAACACCGCTTCCG<br>GCAGCAATGAGGACGAGGACGAGACACCCCTCGATCCCGTCGTCGTCATC<br>TCCAACGACAAGAGCACGGAATTCTTTCTGGCCACGGTCGTCGAGGAAGC<br>AGCCGCGGTGGCGCAAGCCCAGCTGAGCGGGACGCCCCACGTTCGCAAAG<br>ATTCCGGTTACGACGGCGCGGCGAGCAGCACGATGATCCACGAACCCCTC<br>GAGACGAATTCCAGCCCGAGCCCGAACCCGCGGATCACCTCGGCTCCGTC<br>GTCGTCGACCTCGTCGTCGCCGCCGCCGACGAGAGGTGCGACCAGCGTCT<br>CGTCGCAGACGAAGAAGAACGGCAACGGCAGCACGAACAAGCAGGAGCTC<br>AAGAGACTGAAGAGTGCCGGCTCGCTATTGCCGCTGCAGCTCGCGAGGAC<br>GCCCAGCGTGCTGTCGTCCGCCGGCAAGAAGGACCGCAAGAACGCCTCGG<br>CCGGGTCAAGGTTCACGATATACAAGGCCAACAAGGCCAGCAAAAAGAAG<br>AGAGAGAAGAGTTCGGCCAAGAAGGAGCGCAAGGCCACGAAGACTCTAGC<br>GATCGTGTTAGGGGTCTTTCTGATATGCTGGGTACCCTTCTTCACCTGCA<br>ACATCATGGATGCAATCTGCACGAAACTGACGAAGGCCTGTCAGCCTGGT<br>GTTACAGCTTTTATCATCACCTCCTGGTTGGGTTATATGAACAGCTTTGT<br>GAATCCCGTAATATACACTGTGTTCAACCCCGAGTTCCGCAAGGCCTTCC<br>GCAAGTTGATCAGCGTGTAA |

**Table S3.** Dopamine receptor protein sequences

| | |
|---|---|
| ***Hsal*Dop-1** | MTFAGVNDLLGYWVFGLWFCDIWIAFDVMCSTASILNLCAISLDRYIHIKDPLRYGRWV<br>TRRVAIGGIAVVWLLAGLISFVPISLDLHRADQPALYNDGIEEHPTCALDITPTYAVVSSC<br>ISFYVPCIVMLGIYCRLYCYAQKHVRSIRAVTKLPDTSMAKSFRSKSSRCKPPKPQTKT<br>KPTSPYHVSDHKAAITVGVIMGVFLICWVPFFCVNIVAAYCKTCIPLRAFQVLTWLGYSN<br>SAFNPIIYSIFNTEFREAFKRILTKGARARGNQPSTSECGEFRSVVVQKRNGSMVECNI<br>SPRSSADSCQVGVMAQRHRDTIVSAI |
| ***Hsal*Dop-2** | MNDSEIYLLSWEDEVHATNNDDLGRSFYNASYPPFNSSYENLWELATDRAGLAIVLLLF<br>SVATVFGNTLVILAVFRERYLHTATNYFVTSLAFADCLVGLVVMPFSAVYEVLENRWLF<br>TTDWCDVWRSLDVLFSTASILNLCVISLDRYWAITDPFTYPTRMSRKRAAILIAIVWICSS<br>AISFPAIAWWRAVRTEQVPEDKCPFTENLGYLIFSSTISFYLPLFVMVFTYYRIYRAAVIQ<br>TRSLKLGTKQVMMASGELELTLRIHRGGGTNTDARHLFRTTSSTPEELQDLEEPLTALH<br>NNGLTRVPSARHTINNKQHLGKNFSLSRKLAKFAKEKKAAKTLGIVMGVFIICWLPFFVV<br>NLWSGFCTRCIWQEEIVSAAVTWLGWINSGMNPVIYACWSRDFRR |
| ***Hsal*Dop-3** | VFLQVNGSWSLPGFVCDFYIAMDVTCSTSSIFNLVAISIDRYIAVTQPIKYAKHKNNRRV<br>WLTILLVWAISAAIGSPIVLGLNNTPDRIPDQCLFYNTDFIIYSSLSSFYIPCIIMVFLYYNIF<br>KALRNRARRARASKKPNLGDIKPGSIIENIAHTRRFAETALGAAALVAPGIEEPTNTASG<br>SNEDEDETPLDPVVVISNDKSTEFFLATVVEEAAAVAQAQLSGTPHVRKDSGYDGAAS<br>STMIHEPLETNSSPSPNPRITSAPSSSTSSSPPPTRGATSVSSQTKKNGNGSTNKQEL |

| | |
|---|---|
| | KRLKSAGSLLPLQLARTPSVLSSAGKKDRKNASAGSRFTIYKANKASKKKREKSSAKKE<br>RKATKTLAIVLGVFLICWVPFFTCNIMDAICTKLTKACQPGVTAFIITSWLGYMNSFVNPV<br>IYTVFNPEFRKAFRKLISV |