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Effects of Octopamine on Reproduction, Juvenile Hormone Metabolism, Dopamine, and 20-Hydroxyecdysone Contents in *Drosophila*

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The effect of an experimentally increased octopamine content (feeding flies with OA) on the levels of juvenile hormone (JH) degradation, dopamine (DA), and 20-hydroxyecdysone (20E) contents, oogenesis, and fecundity of wild type *Drosophila* flies has been studied. OA feeding of the flies was found to (1) cause a considerable decrease in JH degradation in females, but not males, of *D. melanogaster* and *D. virilis;* (2) have no effect on DA content in *D. melanogaster* and *D. virilis;* (3) increase 20E contents in *D. virilis* females; (4) decrease to a large extent the number of vitellogenic (stages 8–10) and mature (stage 14) oocytes in *D. virilis;* and (5) decrease the fecundity of *D. melanogaster* and *D. virilis*. A possible mechanism of action of OA as a neurohormone on the reproductive function of *Drosophila* is discussed. Arch. Insect Biochem. Physiol. 65:000–000, 2007.

KEYWORDS: *Drosophila*; octopamine; juvenile hormone; dopamine; 20-hydroxyecdysone; reproduction

INTRODUCTION

Octopamine (OA), one of the key insect biogenic amines, is known to work as (1) a neurotransmitter passing the nerve impulse across the synaptic cleft, (2) a neuromodulator affecting the neighboring cells of the CNS locally, which modifies neurotransmitter action, and (3) a neurohormone that is transported with the flow of hemolymph and acts at greater distances (for reviews see Evans and Octopamine, 1985; Roeder, 1999).

It has been shown by a series of studies that OA, as a neurotransmitter/neuromodulator, is involved in the regulation of *Drosophila* reproductive function by controlling the processes of ovulation and oviposition (Monastirioti et al., 1996; Monastirioti, 2003; Cole et al., 2005). For instance, females of the *D. melanogaster* strain $T\beta h^{nM18}$, which are octopamineless as a result of a null mutation of the gene *tyramine-β-hydroxylase* (*Tβh*) that encodes the enzyme converting tyramine into OA, accumulate fully developed eggs in the ovaries, but fail to release them into the oviducts. The reason is, apparently, the inability of the oviduct muscles to contract because of impairment of the nerve-muscle interactions in the absence of OA (Monastirioti et al., 1996; Monastirioti, 2003). Females of another *D. melanogaster* strain with the mutation in the *dTdc2* gene that controls the synthesis of the neural form of tyrosine decarboxylase, the first enzyme involved in the OA synthesis, lack neural tyramine and OA and are unable to deposit eggs

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although they release them into the oviducts (Cole et al., 2005).

OA acting as a neurohormone should also affect insect reproductive function because a change in the OA content, either experimental (Lafon-Cazal and Baehr, 1988; Thompson et al., 1990; Woodring and Hoffmann, 1994; Kaatz et al., 1994; Rachinsky, 1994) or caused by a mutation (Gruntenko et al., 2000), has been shown to lead to changes in juvenile hormone (JH) synthesis and degradation rates. Thus, exogenous OA was shown to inhibit in vitro JH synthesis and its release from corpora allata in females of *Diploptera punctata* and in *Gryllus bimaculatus* (Thompson et al., 1990; Woodring and Hoffmann, 1994). In contrast, OA was found to stimulate in vitro JH production by the corpora allata in larvae and adults of *Apis mellifera,* and in males of *Locusta migratoria* (Lafon-Cazal and Baehr, 1988; Kaatz et al., 1994; Rachinsky, 1994). Such different effects of OA on corpora allata may be due either to interspecies differences or to the diverse effects of OA on the JH system in different sexes and at different developmental stages. It was also established that OA might have a regulatory action not only on JH synthesis, but also on its degradation: exogenous OA increased the activity of the JH-esterase in larvae of *Bombyx mori* and pupae of *Tribolium freemani* (Hirashima et al., 1999b). Our study (Gruntenko et al., 2000) established that young and mature octopamineless females of the *D. melanogaster* strain $T\beta h^{nM18}$ have a sharply increased JH degradation level. This led us to the supposition that OA regulates in vivo JH metabolism and thus controls *Drosophila* reproductive function acting as a neurohormone (Gruntenko and Rauschenbach, 2004).

Here we study the mechanism of this regulation examining the effects of exogenous OA on JH degradation, dopamine (DA), and 20-hydroxyecdysone contents, oogenesis, and fecundity of wild type *D. melanogaster* and *D. virilis* individuals and demonstrate that the experimental increase in the OA content results in a fecundity drop, which may be due to the increase of the ecdysteroid titers in females.

MATERIALS AND METHODS

Maintenance of Stocks

Two species of *Drosophila* were used: *D. virilis* (wild type strain 101 (*wt*)) and *D. melanogaster* (wild type strain *Canton S*). Cultures were raised on standard medium (Rauschenbach et al., 1987) at 25°C at a density of 20 larvae/7 ml of medium, and adults were synchronized at eclosion.

JH Hydrolysis Assay

JH hydrolysis was measured by the partition assay of Hammock and Sparks (1977). Each fly was homogenized in 30 µl of ice-cold 0.1 M sodium-phosphate buffer, pH 7.4, containing 0.5 mM phenylthiourea. Sample size varied from 5 to 8 individuals for each group. Homogenates were centrifuged for 5 min at 13,030*g*, and samples of the supernatant (10 µl) were taken for the assay. A mixture consisting of 0.1 µg of the unlabeled JH-III (Fluka, Buchs, Switzerland) and 12,500 dpm [3H]-JH-III labelled at C-10 (17.4 Ci/mmol, NEN Research Products, Rodgau-Jugesheim, Germany, additionally purified before use) was used as a substrate. The reaction was carried out in 100 µl of the incubation mixture for 30 min, and was stopped by the addition of 50 µl of a solution containing 5% ammonia, 50% methanol (V/V), and 250 µl of heptane. The tubes were shaken vigorously and centrifuged at 13,030*g* for 10 min. Samples (100 µl) of both organic and aqueous phases were placed in vials containing dioxane scintillation fluid; radioactivity was estimated by liquid scintillation counting (Rackbeta 1209 counter, Vellag, Turku, Finland, at 67% counting efficiency for tritium). Control experiments have shown a linear substrate-reaction product relationship; the activity measured is proportional to the amount of supernatant (i.e., enzyme concentration) (Gruntenko et al., 1999; 2000).

DA Content Measurements

Flies were homogenized on ice in 0.1 M $HClO_4$. The homogenates were centrifuged for 10 min at

13,030*g*. The supernatant was filtered through a nylon filter (Schleicher&Schuell, Spartan 3 mm/0.45 µm, Dassel, Germany) and 10 µl was injected directly into an HPLC column through a valve fitted with a 20-µl sample loop. Chromatography was carried out in a C16 reverse-phase column (Diaspher-110-C16, 2.1×150 mm, 5 µm average particle size, BioChemMak, Russia) using an Agilent 1100 HPLC system, with a quaternary pump (including vacuum degasser) and thermocontrolled column compartment. Separated compounds were detected simultaneously by a variable wavelength detector (10-mm path length, 13-µl cell volume) set at 196 nm. Signals from the UV detector were recorded and integrated by a PC using the manufacturer's software. The flow rate was maintained at 0.4 ml/min, the mobile phase consisted of 0.025 M KH_2PO_4 (pH 3.0) buffer, 0.3 mM sodium heptanesulfonate as an ion-pair reagent and 1.5% (v/v) acetonitrile. The concentration of DA was calculated by comparing the peak area between the sample and standard. The identities of the DA and tyrosine peaks in *Drosophila* samples were confirmed on the basis of comparison of their retention times with those of the standard mixture. UV-spectra of the peaks in *Drosophila* samples corresponded to UV-spectra of tyrosine and DA. Moreover, we added excess quantities of DA and tyrosine (20 and 100 ng, respectively) to some *Drosophila* samples to register the changes of heights of the appropriate peaks. Sample size varied from 5 to 6 measurements for each group.

20E Content Measurements

Flies (30 *D. virilis* individuals or 50 *D. melanogaster* ones) were homogenized in 500 µl of 100% methanol containing 400 pg of 25-S-inokosterone (kindly provided by Prof. V. Volodin, Institute of Biology Russian Academy of Sciences, Siktivkar, Russia) as internal standard. Homogenate was heated in a water-bath (60°C) for 10 min and centrifuged at 13,030*g* for 10 min; supernatant was transferred to a glass tube. Residue was extracted repeatedly by 500 µl of methanol and the extracts were combined. One milliliter of the final extract was diluted with 4 ml of the double-distilled water and extracted twice by 5 ml of chloroform in a separating funnel. Water-methanol phase was applied onto a 3-ml disposable C16 extraction column (Diapack-C16, BioChemMak ST, Moscow, Russia). The fluid was drawn through the column using a 5-ml syringe, and ecdysteroids were eluted from the extraction column with 5 ml 65% methanol. The eluate was rotary concentrated to dryness at 60°C and resuspended twice in 500 µl of 100% methanol. The final methanol extract was concentrated to 50 µl, and 10 µl was injected onto an HPLC C16 column (Diaspher-C16, BioChemMak ST, Moscow, Russia, 2×150 mm, pore size 110A, particle size 5 µm), the mobile phase was 15% acetonitrile in water and the flow rate was 0.5 ml/min. 20-hydroxyecdysone and 25-S-inokosterone concentrations were determined by HPLC with a quadrupole mass-spectrometric detection system (Agilent 1100 Series LC/MSD VL, Palo Alto, CA) in the SIM mode according to Wainwright et al. (1997). Sample size varied from 5 to 6 measurements for each group. **AQ1**

Fecundity Analysis

Fecundity analysis was designed as follows: 3 newly eclosed females and 3 males were placed into vials (10 vials for *Canton S* strain and 20–30 vials for *wt* strain in each control and experimental series), in which the bottom and 1 cm of the wall were covered with filter paper soaked with 0.5 ml of the nutrition medium, which contained 0.5% sucrose and 0.2% yeast. In the experimental series, 2.5 or 5 mg of OA (Sigma) were added to this solution. Flies were transferred to vials with fresh medium daily for 9 days. Fecundity was determined as a number of eggs laid by a female within 24 h (the number of eggs laid in each vial was related to the number of females held in the vial for 24 h).

Preparation of Ovaries and Determination of Oocyte Stages

Ovaries were dissected in Ringer's solution (130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 50 mM **AQ2**

Na2HPO4, pH 7.0), fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 20 min and then washed in PBS for 2 × 5 min to remove the fix. Ovaries were further dissected into single ovarioles and then mounted for viewing with phase contrast optics on a Zeiss Axioskop 2 Plus microscope. Images were stacked and manipulated with Axio Vision software. The oocytes were staged according to King (1970). Oocyte stages were counted for 9 pairs of ovaries in the control and for 11 pairs in OA-treated groups.

Statistical Analysis

The significance of the differences between the data sets was tested by the Student's *t*-test

RESULTS

Effect of OA Treatment on Fecundity in *wt* and *Canton S* Flies

Figure 1 shows the results of the fecundity of *D. virilis* (Fig. 1A) and *D. melanogaster* (Fig. 1B) wild type strains raised in vials where OA (doses: 2.5 and 5 mg amine per vial) was added for 9 days compared to their respective control groups. It is clear that OA feeding does not affect the time of the oviposition onset. However, starting from the first day after the oviposition onset (day 4 after eclosion in *D. virilis* and day 2 in *D. melanogaster*), the experimental increase of the OA content leads to a dose-dependent drop in fecundity in both spe-

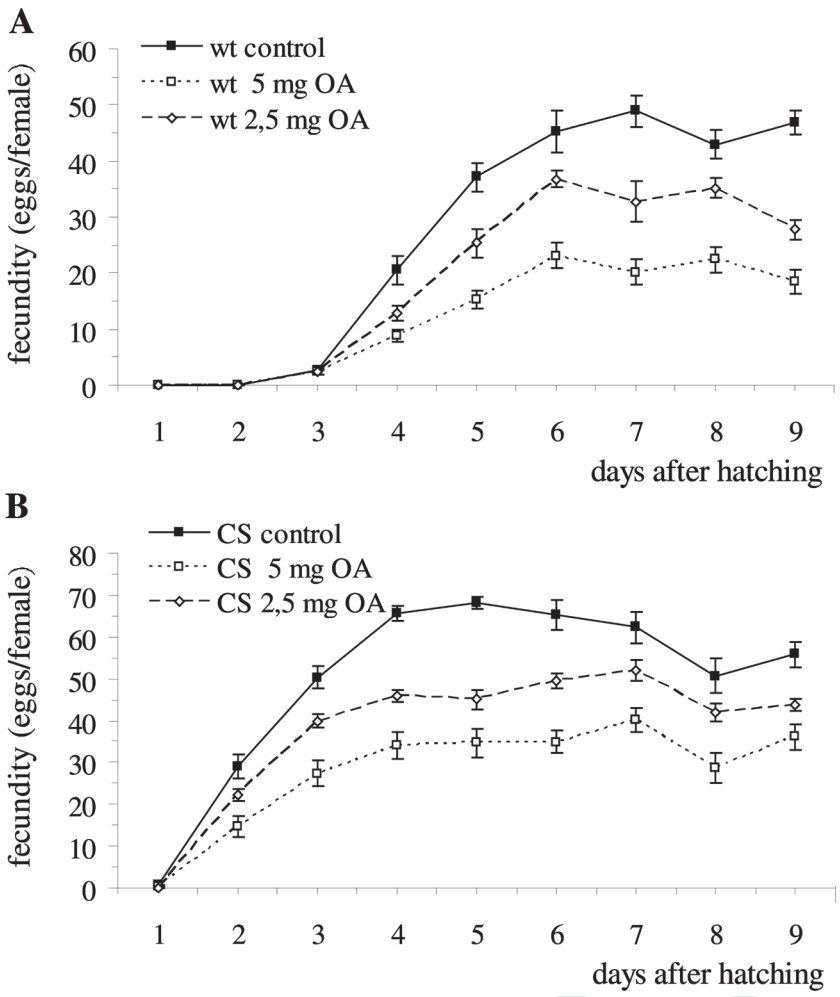
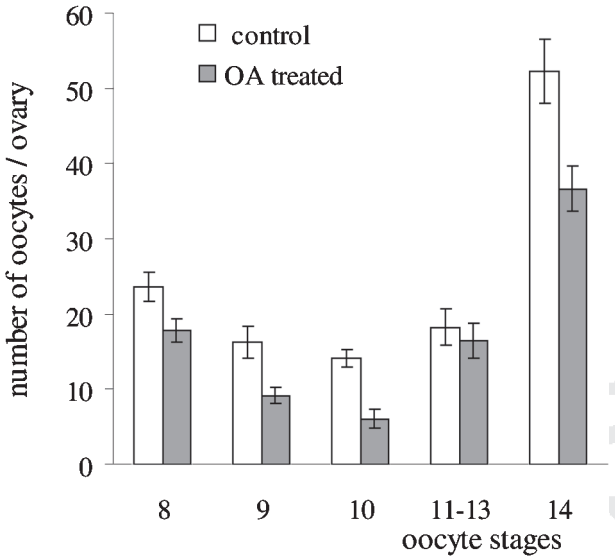


Fig. 1. The effect of the experimentally increase of the OA level on the fecundity of flies of the *D. virilis* wild type strain *wt* (**A**) and *D. melanogaster* strain Canton S (**B**). Means ± SE

cies. Under a 5-mg dose, the differences between OA-treated and control flies are significant at $P < 0.001$ for both strains at every experiment day. Under the 2.5-mg dose, the differences between OA-treated and control flies are significant at $P < 0.05$ on days 4, 6, and 8 after eclosion and at $P < 0.01$ on the other days for *D. virilis* and at $P < 0.05$ on days 2, 7, and 8, at $P < 0.01$ on days 3 and at $P < 0.001$ on the other days for *D. melanogaster*. The fecundity differences between groups treated by different OA doses are significant at $P < 0.05$ on day 4, at $P < 0.01$ on days 7, 9, and at $P < 0.001$ on the other days for *D. virilis* and at $P < 0.05$ on day 2, at $P < 0.01$ on days 5, 7, 9, and at $P < 0.001$ on the other days for *D. melanogaster*.

Effect of OA Treatment on Oogenesis of *wt* Females

To elucidate the reason for the fecundity drop in *Drosophila* with the increased OA level, we observed oogenesis in 6-day-old females of the *wt* strain maintained on the medium with OA and in the controls. The pattern of stage distribution of oocytes in the treated and untreated *wt* females is given in Figure 2. It is clear that the number of oocytes at stages 8–10 and 14 is reduced (significant at $P < 0.05$ for stage 8 and at $P < 0.001$ for stages 9, 10, and 14). Egg chambers at stage 14 contain mature eggs, which can be fertilized and laid (for reviews see Koeppe et al., 1985; Bownes, 1986). Thus, OA affects the number of oocytes at all stages of oogenesis.



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Effect of OA Treatment on JH Metabolism in *wt* and *Canton S* Flies

Figure 3A presents the results of measurements of the JH-hydrolyzing activity in young (2-day-old) and sexually mature (6-day-old) *wt* females and in 2-day-old *wt* males, both OA treated (doses: 2.5 and 5 mg per vial) and control. It is clear that the increase in OA content leads in both young and mature females to a dose-dependent decrease of JH degradation level (differences from the control group are significant at $P < 0.001$ for the dose of 5 mg and at $P < 0.01$ for that of 2.5 mg for both young and mature females; differences between doses are significant at $P < 0.01$ for both young and mature females). The increase in OA content has no effect on JH degradation levels in *wt* males.

The effect of exogenous OA on JH-hydrolyzing activity in young (1-day-old) females and males of *Canton S* strain is shown in Figure 3B. One can see that in the OA-treated *Canton S* females, as in *wt* females, the levels of JH degradation decrease in a dose-dependent manner (differences from the control group are significant at $P < 0.001$ for the dose of 5 mg and at $P < 0.01$ for that of 2.5 mg). Like in *wt* strain, OA treatment has no effect on JH degradation levels in *Canton S* males. In summary, OA leads to less JH degradation and will subsequently lead to higher JH titres in females.

Effect of OA Treatment on DA Content in *wt* and *Canton S* Females

The results of DA measurement in young *wt* and *Canton S* females from the control series and those maintained on the medium with OA are shown in Figure 4. It is clear that the presence of OA in the medium does not affect DA content in females of either strain.

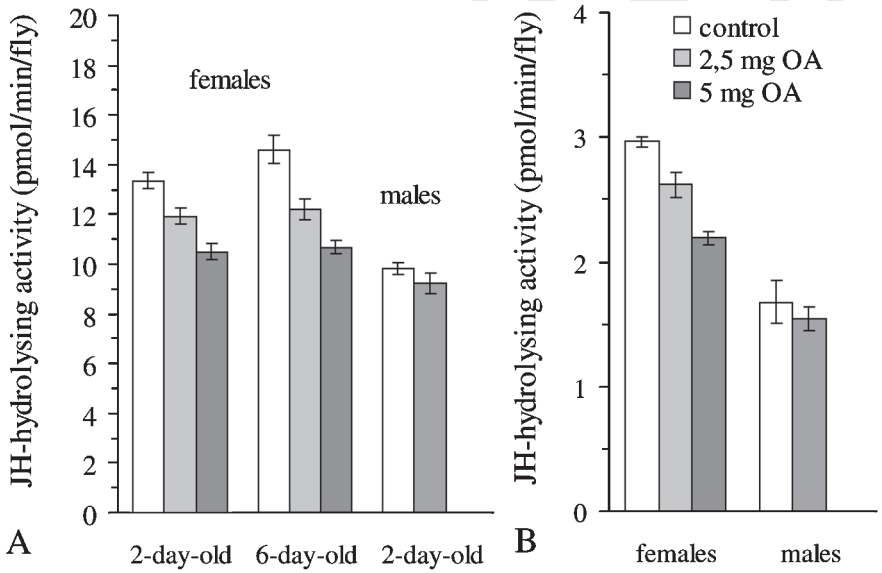

Fig. 3. Effect of OA feeding on JH degradation in 2-day-old females and males and 6-day-old females of *D. virilis* wild type (**A**) and in 1-day-old wild type *D. melanogaster* females and males (**B**). Each point is the average value of 5 to 8 measurements. Means ± SE

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Effects of OA on 20E Contents in *wt* Females of *D. virilis*

The effects of a rise in OA content on 20E level in *wt* females of *D. virilis* are shown in Figure 5. It is clear that the increase in OA content (feeding with the amine) in 2-day-old *wt* females leads to the increase of 20E content (differences from control are significant at $P < 0.01$).

DISCUSSION

The above data indicate that OA, as a neurohormone, can regulate *Drosophila* reproductive function. Indeed, the dose-dependent fecundity drop observed in the OA-treated wild type females of the two *Drosophila* species (see Fig. 1A,B) is not likely to arise from the neuromodulator/neurotransmitter functions of OA, since the lack of ovulation/oviposition, as mentioned above, is related not to an increase, but to a sharp decrease of the OA content (Monastirioti et al., 1996; Monastirioti, 2003; Cole et al., 2005). What is the mechanism by which OA can affect *Drosophila* reproduction?

Earlier we showed that OA, as a neurohormone, controls JH metabolism. In young and mature

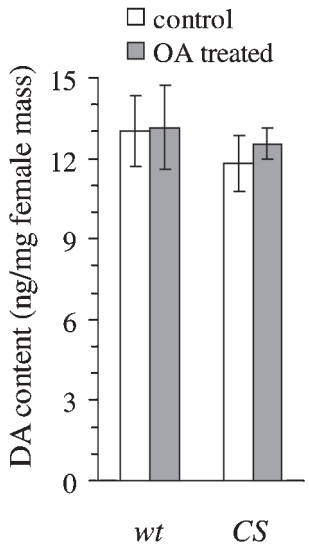

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Fig. 4. Effect of the experimental OA content increase on DA content in 2-day-old *wt* females of *D. virilis* and 1-day-old *Canton S* females of *D. melanogaster*. Each point is an average value of 6 measurements. Means ± SE

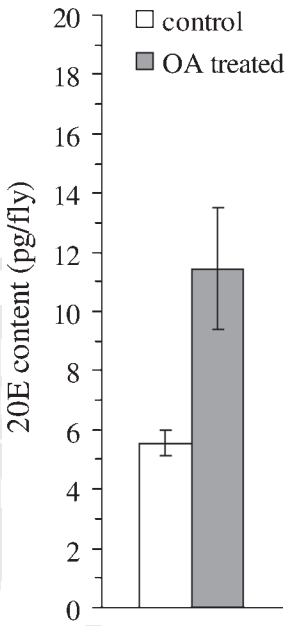

Fig. 5. Effect of OA feeding on 20E level in 2-day-old wild type females of *D. virilis*. Each point is the average value of 5 to 6 measurements. Means ± SE

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octopamineless mutant females of *D. melanogaster*, the levels of JH degradation are increased drastically (Gruntenko et al., 2000), which allowed us to suppose that under normal conditions OA inhibits JH degradation in *Drosophila* females. Our present data provide support for this view (see Fig. 3). In the OA-treated females of both species, JH degradation was decreased by 12 to 26% (doses 2.5 and 5 mg, respectively) in the young *D. melanogaster* females and by 12 to 21% (doses 2.5 and 5 mg, respectively) and 16 to 29% (doses 2.5 and 5 mg, respectively) in the young and mature *D. virilis* females, respectively. The increase in OA content had no effect on JH degradation in males, which agrees with our earlier suggestion (Gruntenko et al., 2005b) that biogenic amines do not play an essential role, as they do in females, in the regulation of JH titre in *Drosophila* males.

Earlier we also showed that JH treatment of *D. virilis* females resulted in a drastic decrease in the hormone degradation (Rauschenbach et al., 2004). We suggested this to be a consequence of the common control of JH synthesis and degradation in *Drosophila*. This suggestion was supported in the study of JH metabolism in ap^{56f} flies. Despite the sharply decreased JH synthesis (Altaratz et al., 1991), ap^{56f} females have steeply increased levels of activity of JH-degrading enzymes (Gruntenko et al., 2003b). The presence of a common control point for JH production and degradation agrees with the fact that in wild type adults of *D. melanogaster*, regulation of JH synthesis and degradation tends to be opposing. Indeed, both JH titer (Bownes and Rembold, 1987; Sliter et al., 1987) and JH synthesis (Altaratz et al., 1991) in young wild type (*¼anton S*) females are substantially higher than in mature flies; at the same time, JH degradation in young *Canton S* females is significantly lower than in the mature ones (Gruntenko et al., 2000, 2003b). The notion of a correlated regulation of JH synthesis and degradation in insects is also supported by the data of Renucci et al. (1990) showing that ovariectomy of *Acheta domesticus* females results in the simultaneous decrease of JH synthesis and an increase in the activity of JH-esterase, which degrades the hormone. Considering all the above, it is reasonable to suppose that the increase of the OA content not only decreases JH degradation in *Drosophila* females (see Fig. 3), but also increases the hormone titre.

Previously, we have revealed (Gruntenko et al., 2000, 2005b) that DA participates in the regulation of JH metabolism as well, inhibiting JH degradation in young *Drosophila* females. It was important to find out whether the decrease of JH degradation observed in OA-treated females (see Fig. 3) had to do only with the OA increase if OA affects DA content and hence changes in DA could be involved in modulating JH metabolism. Theoretically, it is possible since (1) both amines are synthesized from the same source, tyrosine; (2) during regulation of their synthesis there is a feedback system (Gruntenko et al., 2004), whereby an experimental increase in the OA content results in a drop of the activity of tyrosine decarboxylase—the first enzyme in the chain of OA synthesis—and an increase of tyrosine content (Hirashima et al., 2000; Gruntenko et al., 2004). The latter event, in turn, can lead to an increase of DA levels. The data in Figure 4 demonstrate that this is not the case. DA levels in the OA-treated wild type females of both *Drosophila* species are similar to those from control groups. Thus, the change in JH metabolism in the OA-fed females is caused in this case by the increase of the OA, not DA, content. AQ3

There is also a question whether the decrease of fecundity observed upon OA treatment (see Fig. 1) can be caused directly by changes in JH metabolism. Previously, we have shown that the increase in JH titre (the decrease of the hormone degradation not less than by 40%) affects *D. virilis* reproduction by leading to an accumulation of eggs at stage 14 and oviposition arrest. We observed this phenomenon following JH application (JH degradation dropped by 42%) (Rauschenbach et al., 2004), nutrition (Rauschenbach et al., 2004), and heat stress (Gruntenko et al., 2003a) (JH degradation dropped by 40–41%, respectively), as well as upon a dramatic decrease of DA content (JH degradation in mature females dropped by 77%) (Gruntenko et al., 2005b). We observed no oviposition arrest when the flies were fed 20-hydroxy-

ecdysone (20E) (Gruntenko et al., 2005a) or DA (Gruntenko et al., 2005b) (JH degradation dropped by 20–29%, respectively). Therefore, the changes in reproduction upon the increase of OA content (see Figs. 1, 2) that we report here cannot apparently be a direct result of the changes in JH metabolism, since the decrease of its degradation does not exceed 12–29% (see above). This agrees well with the data obtained in the study of oogenesis in the 6-day-old OA-treated females. Stage 14 oocytes accumulation is not observed in these flies, but, on the contrary, there is a decrease in the number of mature oocytes (see Fig. 2). The decreased number of the vitellogenic oocytes at stages 8–10 (see Fig. 2) in the OA-treated females is unlikely to be a direct consequence of changes in JH metabolism. Indeed, earlier we showed that although the experimental increase of JH titre (the hormone application) resulted in *Drosophila* in the oviposition arrest for 24 h, it did not lead to a fecundity decrease after oviposition had resumed, i.e., to a decrease in the number of vitellogenic oocytes (Rauschenbach et al., 2004).

It has been found that an increase in the 20E titre under stress (Gruntenko et al., 2003a) or upon the exogenous 20E treatment (Soller et al., 1999) results in the degradation of vitellogenic oocytes (Soller et al., 1999; Gruntenko et al., 2003a) and in a fecundity drop in *Drosophila* (Gruntenko et al., 2005a). A rise of 20E titer following the increase in OA level (see Fig. 5) corresponds to our data showing a considerable decrease in 20E in octopamineless $T\beta H^{NM18}$ females of *D. melanogaster* (Rauschenbach et al., 2007). The elevation of 20E titre could be due to the OA-induced increase in the JH titre. Indeed, JH has been shown to stimulate ecdysteroid synthesis in *Drosophila* ovaries (reviewed in Postlethwait and Parker, 1987; Richard et al., 1998). Our data provide support for this view. The increase in JH titre (application of the hormone) in young *wt* females of *D. virilis* results in a sharp increase in 20E level (Rauschenbach et al., 2007). It cannot be ruled out, however, that besides the regulation of 20E titre mediated via a JH system, OA may also regulate 20E titre directly. This possibility is supported by Hirashima et al. (1999a) who show that exogenous OA affects in vitro ecdysteroid synthesis by the prothoracic gland of *Bombyx mori* larvae.

It should be noted that the decrease in the number of oocytes on stage 14 in the OA-treated *Drosophila* females (see Fig. 2) can be due either to the degradation of vitellogenic oocytes (stages 8–10) induced by 20E or to the more intensive ovulation/oviposition as a result of oviduct/uterus stimulation by the increased OA content. The latter seems unlikely as we do not see increased egg laying rates (Fig. 1.)

To conclude, OA regulates *Drosophila* reproduction not only as a neurotransmitter/neuromodulator, but also as a neurohormone, changing the metabolism of gonadotropins.

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