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GENERAL GENETICS

The *ecdysoneless*^{1} Gene Regulates Metabolism of the Juvenile Hormone and Dopamine in *Drosophila melanogaster*

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Abstract—The dopamine (DA) content and the level of juvenile hormone (JH) degradation were studied in females of the wild-type Canton S strain and the *ecdysoneless*^{1} (*ecd*^{1}) mutant, which does not produce ecdysone at a restrictive temperature (29°C). Exposure at the restrictive temperature considerably increased the JH-hydrolyzing activity and the DA content in five-day *ecd*^{1} females compared with flies of both strains growing at 19°C and Canton S females exposed at 29°C. In one-day *ecd*^{1} females, the level of JH degradation also increased at the restrictive temperature, but the DA content was low. The effect of ecdysone deficiency on the stress reaction in *Drosophila melanogaster* females was studied using changes in DA content and JH degradation as the reaction indicators. The *ecd*^{1} mutation did not prevent the initiation of the stress reaction in females exposed at the restrictive temperature, but changed its intensity (stress reactivity). The interaction of 20-hydroxyecdysone with JH and DA in regulating *Drosophila* reproduction under normal conditions and in stress is discussed.

INTRODUCTION

The recessive temperature-sensitive *ecdysoneless*^{1} (*ecd*^{1}) mutation disrupts synthesis of the steroid hormone ecdysone at a restrictive temperature (29°C) and causes various developmental and reproductive defects in *Drosophila melanogaster* [1–5]. The ring gland of larvae ceases producing ecdysone at 29°C, and larvae do not develop into pupae when exposed to the restrictive temperature at the early third instar. However, pupation can be induced at 29°C by adding 20-hydroxyecdysone (20E) to the larval medium [1, 3–5]. When *ecd*^{1} females are exposed at 29°C immediately after eclosion, the ovaries stop producing ecdysone and the females become sterile, because oogenesis is terminated in early vitellogenesis [1, 2, 5]. Yet this effect is reversible: vitellogenesis is resumed at a lower temperature [6]. The effect of the *ecd*^{1} mutation on ecdysone production is autonomic: first, even in vitro the ring gland of mutant larvae ceases producing ecdysone at the restrictive temperature [3–5] and, second, ecdysone production in ovaries of *ecd*^{1} females stops when the mutant ovaries are transplanted to wild-type females and the recipients are incubated at the restrictive temperature [1].

It was commonly believed until recently that vitellogenesis—synthesis of yolk proteins (YPs) and their uptake by oocytes—is regulated both by the juvenile hormone (JH), which is synthesized in *corpus allatum*, and by ecdysteroids, which are produced by ovarian follicular cells and other tissues [7–9]. However, Richard *et al.* [10, 11] have recently advanced a hypothesis that JH initiates only early vitellogenesis and ovarian production of ecdysteroids in *Drosophila*, while the major role in the direct control of oogenesis is played by 20E, which stimulates YP production in the fat body and follicular cells and YP uptake by oocytes. At the same time, Soller *et al.* [12] have experimentally studied the effect of exogenous JH and 20E on vitellogenesis in *D. melanogaster* and concluded that the development of vitellogenetic oocytes, including YP synthesis in follicular cells and YP uptake by oocytes, is stimulated by JH, while 20E regulates the previtellogenetic development of oocytes. It has been assumed that a balance of JH and 20E is essential for normal oogenesis in *Drosophila* [12].

We have studied the effect of changes in the ratio between 20E and JH on oogenesis in *D. virilis* and confirmed this assumption: changes in the contents of edogenous 20E and JH as a result of heat stress, starvation, or a mutation dramatically distort oogenesis [13, 14]. In addition, we have shown that, in stress, 20E regulates early oogenesis, while JH controls late oogenesis and oviposition [13, 14]. A stable shift of the gonadotropin balance in favor of 20E (adding 20E to t strain 101, which becomes comparable in fertility with strain 147 carrying a mutation that increases the level of endogenous 20E [15]. An increase in 20E titer decelerates JH degradation and increases the dopamine (DA) content in young wild-type *D. virilis* females [16]. Since DA regulates JH metabolism in *Drosophila* [17], we assumed that 20E regulates the JH titer indirectly, through the DA system [16].

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To check this assumption, it is possible to study JH and DA metabolism in flies that belong to the same strain (*ecd*^{1}) and either do (at 19°C) or do not (at 29°C) produce ecdysone.

In addition, we have shown earlier that ecdysteroids, biogenic amines, and JH are components of the stress reaction in *Drosophila* [18–23]. We have also shown that neither heat shock response, biogenic amines, nor JH are the triggers of the stress reaction [13, 22, 24]. In this work, we use the *ecd*^{1} mutation to study whether 20E acts as a triggering factor.

MATERIALS AND METHODS

We used two *D. melanogaster* strains, wild-type Canton S and mutant *ecd*^{1}. Exposure of *ecd*^{1} flies at the restrictive temperature (29°C) immediately after eclosion stops ecdysone synthesis and causes both female and male sterility [1, 2, 5].

Flies were reared on a standard medium at 19°C at a density of 20 larvae per 7 ml of the medium. Cultures were synchronized by the hatching of larvae (first-instar larvae were collected) and eclosion of adults (flies were collected for 3–4 h after eclosion). In a test series, flies were transferred into vials and exposed at 29°C immediately after eclosion.

To induce stress, flies were incubated in a thermostat at 38°C for 60 min to study the heat stress reaction of the DA system and for 3 h to study the response of the JH system.

JH-hydrolyzing activity was assayed by Hammock and Sparks' method with modification [25]. Flies were homogenized in 30 μl of 0.1 M Na-phosphate (pH 7.4) on ice. The homogenate was centrifuged at 12 000 rpm for 5 min, and 10-μl aliquots of the supernatant were used for the reaction. The reaction time was 30 min, as experimentally determined earlier [19]. As a substrate, we used a mixture of 12 500 dpm of JH-III tritiated at C-10 (specific activity 11.9 Ci/mmol, NEN Research Products) and 0.1 μg of nonlabeled JH-III (Sigma). The reaction was carried in 100 μl of 0.1 M Na-phosphate (pH 7.4) supplemented with 0.5 mM phenylthiourea at 37°C and terminated by adding 50 μl of an aqueous solution containing 5% ammonia and 50% methanol (v/v). Nonhydrolyzed JH was extracted with 250 μl of heptane. The mixture was thoroughly shaken and centrifuged at 12 000 rpm for 10 min. Aliquots (100 μl) of the aqueous and heptane phases were transferred into vials with a dioxane scintillation medium and tested for radioactivity.

DA content was measured according to Maikel *et al.* [26], with modification. Flies were weighed and homogenized in 0.1 N $HClO_4$ (0.3 ml per fly) on ice. The homogenate was centrifuged at 12 000 rpm for 10 min. The amine content was measured in 0.1 ml of the supernatant (each sample was examined twice). Samples were incubated in a water bath at 96°C for 6 min, immediately chilled on ice, and combined with 1 ml of bidistilled water. Measurements were performed using a Hitachi fluorimeter (Japan). The excitation and emission wavelengths were 330 and 370 nm, respectively.

Statistical analysis of the results was performed with Student's *t* test. The stress reactivity was calculated as a percent change in a trait in each stressed fly relative to a control (each experimental value was estimated relative to the mean value obtained for the control group, because it is infeasible to measure biochemical parameters in the same *Drosophila* individual under normal conditions and in stress).

RESULTS

JH Hydrolysis in Five-Day Females of the ecd^{1} *and Canton S Strains at Permissive and Restrictive Temperatures and after Heat Stress*

To study whether a lack of ecdysone affects JH metabolism, JH-hydrolyzing activity was assayed in *ecd*^{1} and Canton S females developing at 19°C or exposed at 29°C immediately after eclosion (i.e., under the conditions that prevent ecdysone synthesis in *ecd*^{1} flies [1, 2, 5]). In addition, it was of interest to determine whether ecdysone deficiency changes the response of the JH degradation system to a stress factor (38°C). The results of these experiments are shown in Fig. 1.

Exposure at 29°C considerably increased the JH-hydrolyzing activity in *ecd*^{1} females compared with flies of both strains reared at 19°C and with Canton S females exposed at 29°C (differences were significant at $P < 0.001$ for all groups). Note that the level of JH degradation in Canton S females exposed at 29°C did not significantly differ from that in *ecd*^{1} and Canton S females growing at 19°C.

When flies were exposured to a stress factor, the JH system responded by a decrease in JH degradation in Canton S and *ecd*^{1} females both at the permissive and at the restrictive temperature (the difference from the control was significant at $P < 0.01$ for Canton S and at $P < 0.001$ for *ecd*^{1}).

DA Content in ecd^{1} *and Canton S Females at the Permissive and Restrictive Temperatures and in Heat Stress*

The question arose as to whether the change in 20E directly affects JH metabolism or its effect is mediated by the system of biogenic amines, since JH degradation is regulated by DA under normal conditions (DA inhibits JH degradation in young females and stimulates it in old females) [27].

Our results (Fig. 2) showed that the DA level considerably increased in *ecd*^{1} females developing at the restrictive temperature (the differences from females of either line developing at 19°C and from Canton S females exposed at 29°C were significant at $P < 0.001$).

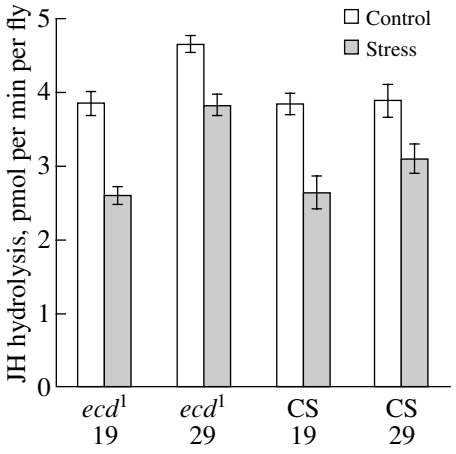

Fig. 1. Hydrolysis of JH in five-day females of the *ecd*[1] and Canton S (CS) strains at the permissive (19°C) and restrictive (29°C) temperatures and in heat stress (38°C, 3 h).

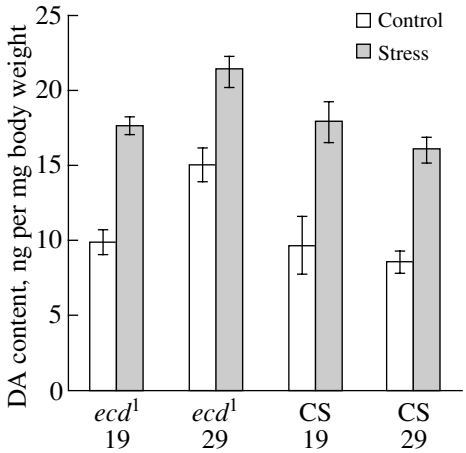

Fig. 2. DA content in five-day females of the *ecd*[1] and Canton S (CS) strains at the permissive (19°C) and restrictive (29°C) temperatures and in heat stress (38°C, 1 h).

Like in the case of JH degradation, *ecd*[1] and Canton S females growing at 19°C and Canton S females exposed at 29°C did not significantly differ in DA content.

Heat stress increased DA content in all groups (the difference from the control was significant at $P < 0.01$ for *ecd*[1] at 29°C and for Canton S at 19°C and at $P < 0.001$ for *ecd*[1] at 19°C and for Canton S at 29°C).

Stress Reactivity of the JH and DA Systems of ecd[1] *and Canton S Females at Permissive and Restrictive Temperatures*

To characterize the response of the JH and DA systems to heat stress under conditions of ecdysone deficiency, we calculated the stress reactivity of these systems for *ecd*[1] and Canton S females exposed at the permissive and restrictive temperatures. The reactivity was obtained as a percent decrease in the level of JH degradation and a percent increase in the DA content in heat stress (38°C) as compared with the corresponding values observed under normal conditions.

As shown in Fig. 3, the stress reactivity of the JH and DA systems was much the same in Canton S flies exposed at 19 or 29°C and in *ecd*[1] females growing at 19°C (the differences between groups were nonsignificant). On the other hand, *ecd*[1] females displayed a considerably lower reactivity ($P < 0.001$ for the DA system and $P < 0.05$ for the JH system) as compared with the other groups.

JH Hydrolysis and DA Content in One-Day Females of the ecd[1] *and Canton S Strains at the Permissive and Restrictive Temperatures*

Since the ontogenetic character of the effects of the hormones under study is well known [17], it was of interest to study whether ecdysone deficiency affects the JH-hydrolyzing activity and DA content in young *Drosophila* females. The results are shown in Fig. 4.

Like in mature females, ecdysone deficiency (at 29°C) significantly ($P < 0.001$) increased the level of JH degradation in young *ecd*[1] females compared with flies having a normal titer of ecdysteroids (at 19°C). However, young *ecd*[1] females displayed the opposite response of the DA system to a dramatic decrease in ecdysone production: exposure at the restrictive temperature decreased the DA content, rather than increasing it as in older females (the difference from *ecd*[1] females growing at 19°C was significant at $P < 0.01$).

DISCUSSION

Postlethwait and colleagues [28–31] have studied in detail the hormonal control of YP synthesis in the ovaries and the fat body of *D. melanogaster.* The following control mechanism was suggested. JH produced in *Corpus allatum* enters the ovaries and stimulates YP synthesis by follicular cells and subsequent YP uptake by oocytes. At the same time, JH induces ecdysone-secreting cells of the ovaries to produce ecdysteroids, which initiate YP synthesis in the fat body and follicular cells of the ovaries [32]. YPs are transferred into oocytes from follicular cells in early vitellogenesis and from the fat body in late vitellogenesis. In the latter case, YPs are secreted into hemolymph and deposited in nursing cells to be then delivered to oocytes through circular channels [33]. Richard *et al.* [10] have confirmed JH-dependent stimulation of ecdysteroid synthesis in the *D. melanogaster* ovaries: biosynthesis of ecdysteroids increases when ovaries obtained from young females (in the first 18 h after eclosion) are incubated with JH bisepoxide-3. In addition, it has been shown that a dramatic decrease in JH in the *apterous*[56f] (*ap*[56f]) *D. melanogaster* mutant (i.e., decreased synthesis [34] and increased degradation [35] of JH) does not

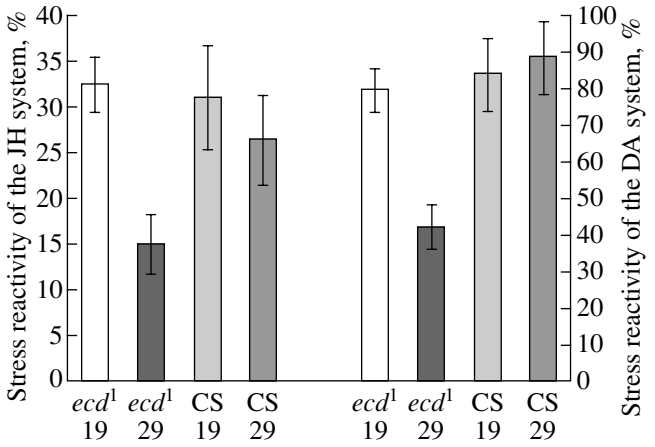

Fig. 3. Stress reactivity of the JH and DA systems in five-day females of the *ecd*^{1} and Canton S (CS) strains at the permissive (19°C) and restrictive (29°C) temperatures.

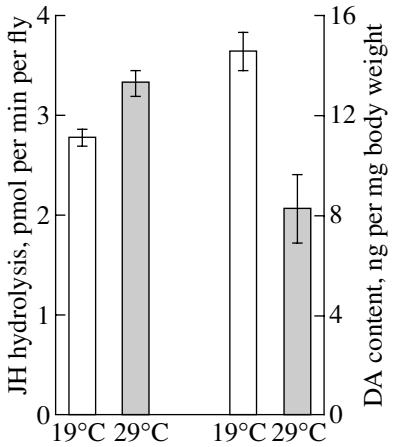

Fig. 4. JH hydrolysis and DA content in one-day *ecd*^{1} females at the permissive (19°C) and restrictive (29°C) temperatures.

reduce the 20E titer. Quite the reverse, *ap*^{56f} females are characterized by considerably more intense synthesis and an elevated content of ecdysteroids in the ovaries during the first three days after eclosion [10].

We believe that the above data suggest reciprocal control of JH and 20E in *Drosophila*: not only does JH stimulates ecdysteroid synthesis, but 20E, in turn, regulates the JH level. Therefore, the high content of ecdysteroids in the *ap*^{56f} mutant can be considered as a compensatory response induced by a decreased JH content and aiming at restoring it.

The results of this work support this assumption: both one- and five-day *ecd*^{1} females exposed at 29°C showed a significantly higher level of JH degradation as compared with females growing at 19°C (Figs. 1, 4). The effect of 20E on JH metabolism is also supported by a decrease in JH degradation in young wild-type *D. virilis* females fed on a medium supplemented with 20E [16].

Another evidence for the interaction of ecdysteroids with JH is provided by the data on stress reactivity of the JH system. The stress reactivity of the JH system in females with ecdysone deficiency is dramatically lower than in females with the normal level of ecdysteroids (Fig. 3).

Biogenic amines—octopamine (OA) and DA—are known to affect both synthesis and degradation of JH in various insects [36–40]. We studied JH degradation in *D. melanogaster* females of the *Tβh*^{nM18} strain, which lacks OA, and in two independent strains carrying the *ebony* mutation, which doubles the DA content. Young and mature females lacking OA showed a dramatically increased level of JH degradation [27] and, probably, had a lower level of JH synthesis, because JH synthesis and degradation change oppositely and are under a common control [35, 41]. At the same time, when the DA content is doubled, the level of JH degradation is dramatically decreased in young females (while JH synthesis is probably increased) and elevated in mature females (while JH synthesis is probably decreased) [27]. Based on these data, we concluded that DA and OA regulate JH metabolism in *Drosophila.* OA inhibits degradation and stimulates synthesis of JH both in young and in mature females, while the character of DA action varies during ontogeny: DA inhibits degradation and stimulates synthesis of JH in young females and exerts an opposite effect in mature females [17, 27, 35]. There is a feedback in this regulation: application of JH decreases the DA level in young females and increases it in mature females of *D. virilis* and *D. melanogaster* [42].

In view of the above, it is logical to assume that the effect of 20E on JH metabolism in *Drosophila* is mediated by the system of DA metabolism. Our results support this assumption. In the absence of ecdysone, the DA content increased in mature females (Fig. 2) and decreased in young females (Fig. 4). Such changes were expected to increase the JH-hydrolyzing activity, and such an increase was observed indeed (Figs. 1, 4). The effect of 20E on the DA content in *Drosophila* is also evident from our previous data that the DA content significantly increases in response to an experimental increase in 20E content in young wild-type *D. virilis* females [16].

It is important to note that our findings support the assumption of Soller *et al.* that a balance of JH and 20E is essential for vitellogenesis in *Drosophila* [12]. At the same time, our results agree with the hypothesis of Richard *et al.* that 20E plays the major role in this process (in particular, regulating the JH titer) [10].

To identify the mechanism triggering the stress reaction, several *D. melanogaster* and *D. virilis* strains with mutations dramatically changing the DA and OA contents, synthesis of heat shock proteins, and JH reception have been studied in our laboratory [18–23]. It was found that the mutations do not prevent the stress reaction, but change its intensity. If 20E were a triggering factor, *ecd*^{1} flies would not develop the stress reaction at the restrictive temperature. Our results demonstrate

that this is not the case: although JH and DA metabolism is changed in *ecd*^{1} flies at the restrictive temperature, the stress reaction is still initiated and involves an increase in DA content in young and mature females and a decrease in JH hydrolysis (Figs. 1, 2, 4). However, the stress reactivity of both systems is changes in *ecd*^{1} flies (Fig. 3), which supports again our previous conclusion that the interaction of stress-related hormones in stress is genetically determined [15].

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