ID JEXBIO/2014/106815

Disruption of insulin signalling affects the neuroendocrine stress reaction in *Drosophila* females

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Abstract

Juvenile hormone (JH) and dopamine are involved in the stress response in insects. The insulin/insulin-like growth factor signalling pathway has also recently been found to be involved in the regulation of various processes, including stress tolerance. However, the relationships among the JH, dopamine and insulin signalling pathways remain unclear. Here, we study the role of insulin signalling in the regulation of JH and dopamine metabolism under normal and heat stress conditions in *Drosophila melanogaster* females. We show that suppression of the insulin-like receptor (InR) in the *corpus allatum*, a specialised endocrine gland that synthesises JH, causes an increase in dopamine level and JH-hydrolysing activity and alters the activities of enzymes that produce as well as those that degrade dopamine (alkaline phosphatase (ALP), tyrosine hydroxylase (TH) and dopamine-dependent arylalkylamine N-acetyltransferase (DAT)). We also found that InR suppression in the *corpus allatum* modulates dopamine, ALP, TH and JH-hydrolysing activity in response to heat stress and that it decreases the fecundity of the flies. JH application restores dopamine metabolism and fecundity in females with decreased InR expression in the *corpus allatum*. Our data provide evidence that the insulin/insulin-like growth factor signalling pathway regulates dopamine metabolism in females of *D. melanogaster via* the system of JH metabolism and that it affects the development of the neuroendocrine stress reaction and interacts with JH in the control of reproduction in this species.

Keywords: *Drosophila*, insulin like receptor, juvenile hormone, dopamine, heat stress

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Introduction

It is well known that maintenance of biological organisms under unfavourable conditions causes the development of non-specific neuroendocrine stress reactions. In insects, the components of such stress reactions include juvenile hormone (JH) and 20-hydroxyecdysone (20E), which play a gonadotropic role in adults, and the biogenic amines dopamine (DA), serotonin and octopamine (OA), which function as neurotransmitters, neuromodulators and neurohormones (Orchard and Loughton, 1981; Davenport and Evans 1984; Rauschenbach et al., 1987, 1993, 1995a, 2004; Woodring et al., 1988; Cymborowski, 1991; Hirashima and Eto, 1993; Neckameyer and Weinstein, 2005; Andersen et al., 2006; Lalouette et al., 2007; Roesijadi et al., 2007; rev.: Gruntenko and Rauschenbach, 2008).

In earlier work, we characterised the development of the neuroendocrine stress reaction in adults of *Drosophila virilis* and *Drosophila melanogaster*. In these species, DA content increases sharply as early as 15 min after the beginning of stress exposure (38°C) (Rauschenbach et al., 1993; Hirashima et al., 2000b; Gruntenko et al., 2004). The increase in DA content is followed by a sharp decrease in the activity of the key enzyme of its synthesis, tyrosine hydroxylase (TH), and this decrease continues for up to 60 min after the onset of stress exposure (Rauschenbach et al., 1995b). A similar pattern is observed with the OA system, the only difference being that the decrease in activity of tyrosine decarboxylase, the first enzyme involved in OA synthesis, continues for up to 240 min of the stress exposure (Sukhanova et al., 1997; Hirashima et al., 2000b). The activity of alkaline phosphatase (ALP, the enzyme that regulates the pool of the DA and OA precursor tyrosine (rev.: Wright, 1987)) also decreases abruptly under stress conditions (Sukhanova et al., 1996). The JH metabolic system responds to heat stress by decreasing JH degradation (Rauschenbach et al., 1995a, 2004; Gruntenko et al., 2000, 2003b). The ecdysteroid system of wild-type flies responds to a 60-min heat stress by an increase in 20E levels, and this increase continues for up to 180 min after the onset of stress exposure (Hirashima et al., 2000a; Gruntenko et al., 2003a).

In the last decade, study of the insulin/insulin-like growth factor signalling pathway (IIS) has been a focus of special interest for many researchers. In *Drosophila*, this pathway contributes to the regulation of various functions, including growth, development, reproduction, metabolic homeostasis, longevity and stress resistance (Tatar et al., 2001, 2003; Clancy et al., 2001; Rulifson et al., 2002; Belgacem and Martin, 2002, 2006, 2007; Broughton et al., 2005; Shingleton et al., 2005; Piper et al., 2008; Gulia-Nuss et al., 2011). The *Drosophila* IIS has been shown to include eight insulin-like peptides as well as DILP1–8, a transcription factor of the Forkhead box class O

family (dFOXO), an insulin-like receptor (InR), and the fly orthologue of mammalian insulin receptor substrates, CHICO (revs: Toivonen and Partridge, 2009; Kannan and Fridell, 2013).

The existing data suggest that interactions occur between 20E, JH and IIS components,. InR expression has been demonstrated in the *corpus allatum* (*CA*), a JH-producing gland (Belgacem and Martin. 2006, 2007). A mutation in the gene *InR* was shown to lead to a decrease in 20E and JH production in *Drosophila* adults *in vitro* (Tatar et al., 2001, 2003; Tu et al., 2002, 2005). A mutation of the gene *chico*, when present in the homozygous state, results in an increase in the titre of ecdysteroids in the haemolymph of 4-day-old *D. melanogaster* females when normalised with respect to body size (Richard et al., 2005). Ecdysone was found to counteract the growth-promoting action of IIS (Colombani et al., 2005).

Considering the above, we suggest that IIS could be one of the components of the neuroendocrine stress reaction in *Drosophila* and that it could affect the development of this reaction. To verify this suggestion, we studied the effects of decreased expression of the *InR* gene in the *CA* on fecundity and on DA and JH metabolism under normal and heat stress conditions in females of *Drosophila*.

Results

InR expression is decreased in the corpus allatum of Aug21-Gal4/p[UAS-RNAi-InR] females

We previously demonstrated specific expression of an Aug21-Gal4 driver in *CA* of *D. melanogaster* adults (Gruntenko et al., 2012b). Here, we used its recombination with *P{UASp-Act5C.mRFP}* to enable visualisation of *CA* and then crossed *Aug21-Gal4; UAS-RFP* flies with a *p[UAS-RNAi-InR]/CyO::arm-GFP* strain carrying the *pUAS-RNAi-InR* interference transgene to downregulate InR expression in the gland. Fig. 1 shows the results of an immunohistochemical assay of *CA* of 1-day-old *Aug21-Gal4/p[UAS-RNAi-InR];UAS-RFP* and *Aug21-Gal4;UAS-RFP* females using anti-InR antibodies. InR immunoreactivity (green fluorescence) is readily detectable in *CA* cells of control *Aug21-Gal4;UAS-RFP* females (Fig. 1a,a_1). In contrast, reduced InR immunoreactivity is observed in the *CA* of *Aug21-Gal4/p[UAS-RNAi-InR];UAS-RFP* females (Fig. 1b,b_1).

Note that 1-day-old *Aug21-Gal4/p[UAS-RNAi-InR]* females are smaller (Fig. 1c) and have lower body weights (Fig. 1d) than control *p[UAS-RNAi-InR]/CyO::arm-GFP* females (differences are significant at $p<0.001$).

Decreased expression of InR in CA results in an increase in JH degradation and an increase in the response of the JH degradation system to heat stress

We studied the effect of decreased InR expression in *CA* cells on JH metabolism under normal and heat stress conditions in 1-day-old *Aug21-Gal4/p[UAS-RNAi-InR]* (InR-) females compared with control *p[UAS-RNAi-InR]/CyO::arm-GFP* females (InR+(1)) as well as with females of the parental strains *p[UAS-RNAi-InR]/CyO* (InR+(2)) and *Aug21-Gal4/CyO::arm-GFP* (InR+(3)). The results are shown in Fig. 2A. In females that downregulate InR in *CA* (*i.e.*, *Aug21-Gal4/p[UAS-RNAi-InR]*), JH degradation levels were significantly higher than in the controls. Comparison of InR downregulation and heat stress effects on the JH degradation level by two-way ANOVA with genotype and temperature as fixed factors revealed significant effects of genotype ($F_{(3, 217)}$=33.79, $p < 0.00001$) and heat stress ($F_{(1, 62)}$=133.66, $p < 0.00001$). A significant interaction of these factors ($F_{(3, 217)}$=7.75, $p < 0.0001$) was also found. No significant differences in JH degradation were found in any of the control groups (*p[UAS-RNAi-InR]/CyO::arm-GFP*, *p[UAS-RNAi-InR]/CyO* and *Aug21-Gal4/CyO::arm-GFP*) (Fig. 2A).

To assess the effect of InR downregulation in *CA* on the response of the JH metabolic system to stress, we calculated the stress reactivity of the JH degradation system (percentage decrease in JH degradation level following heat stress relative to its value under normal conditions) in all of the studied groups. The control groups did not differ in JH stress reactivity (17±2 for *p[UAS-RNAi-InR]/CyO::arm-GFP*, 15±1 for *p[UAS-RNAi-InR]/CyO* and 15±1 for *Aug21-Gal4/CyO::arm-GFP*). In contrast, an increase in JH stress reactivity was observed in *Aug21-Gal4/p[UAS-RNAi-InR]* females (26±1) *vs*. controls (one-way ANOVA: $F_{(3, 110)}$=13.37, $p < 0.00001$ with respect to JH stress reactivity).

JH treatment rescues JH degradation and JH stress reactivity in Aug21-Gal4/p[UAS-RNAi-InR] females

In previous work, we showed that the rate of JH degradation is an indicator of the level of hormone production: a decrease in juvenile hormone synthesis leads to an increase in its degradation and in the stress reactivity of the JH degrading system (Gruntenko et al., 2010). To confirm that the changes in the level of JH-hydrolysing activity and JH stress reactivity found in *Aug21-Gal4/p[UAS-RNAi-InR]* females were due to decreased JH production resulting from decreased InR expression in *CA*, we studied the effect of exogenous JH on these parameters. We treated 1-day-old *Aug21-Gal4/p[UAS-RNAi-InR]* females with JH dissolved in acetone and measured their JH-hydrolysing activity. For comparison, *Aug21-Gal4/p[UAS-RNAi-InR]* and

p[UAS-RNAi-InR]/CyO::arm-GFP females were treated with acetone. Half of the individuals of each group under study were exposed to heat stress (Fig. 2B).

The results of these experiments show that JH treatment of *Aug21-Gal4/p[UAS-RNAi-InR]* (InR-) females decreases JH degradation to the level typical of *p[UAS-RNAi-InR]/CyO::arm-GFP* (InR+(1)) females (Fig. 2B). Comparison of the effects of JH treatment and heat stress on JH degradation in JH and acetone-treated *Aug21-Gal4/p[UAS-RNAi-InR]* females by two-way ANOVA with JH treatment and temperature as fixed factors revealed significant effects of JH treatment ($F_{(1, 47)}$=58.23, $p < 0.00001$) and heat stress ($F_{(1, 47)}$=120.02, $p < 0.00001$). Evidence for an interaction of these factors was also found ($F_{(1, 47)}$=9.31, $p = 0.0038$). JH stress reactivity in JH-treated *Aug21-Gal4/p[UAS-RNAi-InR]* females (21±2) also decreased to the level typical of *p[UAS-RNAi-InR]/CyO::arm-GFP* females (21±2), whereas the stress reactivity of acetone-treated *Aug21-Gal4/p[UAS-RNAi-InR]* females (28±1) was significantly higher (one-way ANOVA, $F_{(1, 24)}$=14.30, $p < 0.001$).

Note that acetone treatment of the flies does not eliminate differences in JH degradation levels (Fig. 2B) or JH stress reactivity between *Aug21-Gal4/p[UAS-RNAi-InR]* and *p[UAS-RNAi-InR]/CyO::arm-GFP* females. Both parameters are elevated in females with decreased InR expression in the *CA* (*i.e., Aug21-Gal4/p[UAS-RNAi-InR]* females). The comparison of InR downregulation and heat stress effects on JH degradation levels in acetone-treated females revealed significant effects of genotype ($F_{(1, 53)}$=92.31, $p < 0.00001$) and heat stress ($F_{(1, 53)}$=170.55, $p < 0.00001$). A significant interaction of these factors ($F_{(1, 53)}$=12.84, $p < 0.001$) was also found. The differences in stress reactivity between acetone-treated *Aug21-Gal4/p[UAS-RNAi-InR]* and *p[UAS-RNAi-InR]/CyO::arm-GFP* females are significant (one-way ANOVA, $F_{(1, 24)}$=11.04, p = 0,0026).

Decreased expression of InR in the CA results in reduced fecundity of Drosophila females

In previous work, we showed that another indicator of the level of JH production is fecundity; decreased hormone synthesis due to genetic ablation of a portion of the insect's *CA* cells leads to a dramatic decrease in fecundity (Gruntenko et al., 2010). Fig. 3A shows the results of fecundity evaluation in the *Aug21-Gal4/p[UAS-RNAi-InR]* flies, which exhibits decreased InR expression in *CA*, and in *p[UAS-RNAi-InR]/CyO::arm-GFP, Aug21-Gal4/CyO::arm-GFP* and *p[UAS-RNAi-InR]/CyO* flies. Most notably, *Aug21-Gal4/p[UAS-RNAi-InR]* females show dramatically decreased fecundity. The comparison of InR downregulation and age effects on fecundity by two-way mixed design ANOVA (day after eclosion – repeated measures factor;

genotype – simple factor) revealed significant effects of genotype ($F_{(3, 41)}$=358.69, $p < 0.00001$) and age ($F_{(7, 287)}$=274.64, $p < 0.00001$). A significant interaction of these factors in females has also been found ($F_{(21, 287)}$=24.34, $p < 0.00001$).

To confirm that decreased JH synthesis in females with decreased InR expression in *CA* leads to a decrease in fecundity, we treated *Aug21-Gal4/p[UAS-RNAi-InR]* females with JH dissolved in acetone and measured their fecundity. For comparison, *Aug21-Gal4/p[UAS-RNAi-InR]* and *p[UAS-RNAi-InR]/CyO::arm-GFP* females were treated with acetone. The results of this experiment are shown in Fig 3B. The increased JH levels in *Aug21-Gal4/p[UAS-RNAi-InR]* females significantly increased their fecundity. The comparison of JH and age effects on fecundity by two-way mixed design ANOVA (day after eclosion – repeated measures factor; JH treatment – simple factor) revealed significant effects of JH ($F_{(1, 15)}$=66.40, $p < 0.00001$) and age ($F_{(4, 60)}$=155.91, $p < 0.00001$) on fecundity. A significant interaction of these factors in females was also found ($F_{(4, 60)}$=58.41, $p < 0.00001$).

Decreased expression of InR in CA results in increased DA content and DA stress reactivity and decreased DA-dependent arylalkylamine N-acetyltransferase activity

To assess the effects of decreased InR expression in *CA* on DA level, we quantified DA content in *Aug21-Gal4/p[UAS-RNAi-InR]* (InR-) *vs.* control (*p[UAS-RNAi-InR]/CyO::arm-GFP*) (InR+(1)) flies under normal and heat stress conditions (Fig 4A). *Aug21-Gal4/p[UAS-RNAi-InR]* females had significantly higher DA content than control flies. Comparison of InR downregulation and heat stress effects on the DA levels in females by two-way ANOVA with genotype and temperature as fixed factors revealed significant effects of genotype ($F_{(1, 42)}$=673.73, $p < 0.00001$) and heat stress ($F_{(1, 42)}$=79.02, $p < 0.00001$) on DA content. A significant interaction of these factors ($F_{(1, 42)}$=15.45, $p < 0.001$) was found. The response of DA to stress in females with decreased InR expression in *CA* was increased relative to control flies: the differences in DA stress reactivity between *Aug21-Gal4/p[UAS-RNAi-InR]* and *p[UAS-RNAi-InR]/CyO::arm-GFP* females (26±2 and 18±2, correspondingly) are significant (one-way ANOVA ($F_{(1, 21)}$=8.14, p=0.0096).

Earlier (Rauschenbach et al., 2011), we found that in young females of *Drosophila* JH controls DA content at the level of amine degradation by regulating the activity of DA-dependent arylalkylamine N-acetyltransferase (DAT, the primary enzyme that degrades DA (Wright, 1987)). We have also determined that DAT is not a component of the *Drosophila* stress response (Rauschenbach et al., 1997). Here, we studied the DAT activity of 1-day-old InR- (*Aug21-Gal4/p[UAS-RNAi-InR]*) and InR+(1) (*p[UAS-RNAi-InR]/CyO::arm-GFP*) females

under normal conditions. The results are shown in Fig. 4B. The level of DAT activity in *Aug21-Gal4/p[UAS-RNAi-InR]* females was significantly lower than that in *p[UAS-RNAi-InR]/CyO::arm-GFP* flies (one-way ANOVA ($F_{(1, 36)}$=32.97, $p < 0.00001$)). Treatment of *Aug21-Gal4/p[UAS-RNAi-InR]* females with exogenous JH (Fig. 4C) showed that the decrease in DAT activity is related to the decrease in the endogenous level of the hormone; the activity of the enzyme in JH-treated InR- females (*Aug21-Gal4/p[UAS-RNAi-InR]*) is increased to the level typical of InR+(1) females (*p[UAS-RNAi-InR]/CyO::arm-GFP*). The differences in DAT activity between JH and acetone-treated *Aug21-Gal4/p[UAS-RNAi-InR]* females are significant (one-way ANOVA ($F_{(1, 27)}$=27.20, $p < 0.001$).

Decreased InR expression in the CA results in decreased alkaline phosphatase and tyrosine hydroxylase activities and decreases in the reactivity of these enzymes to stress

The ALP and TH activities of 1-day-old *Aug21-Gal4/p[UAS-RNAi-InR]* and control (*i.e.*, *p[UAS-RNAi-InR]/CyO::arm-GFP*, *p[UAS-RNAi-InR]/CyO* and *Aug21-Gal4/CyO::arm-GFP*) females were measured under normal and heat stress conditions. As shown in Fig 5A and C, a significant decrease in ALP and TH activities was observed in InR- (*Aug21-Gal4/p[UAS-RNAi-InR]*) females. No statistical significance between the ALP or TH activities of the control InR+ groups (*p[UAS-RNAi-InR]/CyO::arm-GFP*, *p[UAS-RNAi-InR]/CyO* and *Aug21-Gal4/CyO::arm-GFP*) under normal conditions was found. Comparison of InR downregulation and heat stress effects on ALP and TH activity levels in females by two-way ANOVA with genotype and temperature as fixed factors revealed significant effects of genotype (($F_{(3, 164)}$=38.84, $p < 0.00001$) on ALP activity and ($F_{(3, 191)}$=12.75, $p < 0.00001$) on TH activity) and heat stress (($F_{(1, 164)}$=200.04, $p < 0.00001$) on ALP activity and ($F_{(1, 191)}$=1205.76, $p < 0.00001$) on TH activity). A significant interaction of these factors in females was also found (($F_{(3, 164)}$=10.80, $p < 0.00001$) for ALP and ($F_{(3, 191)}$=26.11, $p < 0.00001$) for TH).

To determine whether downregulation of InR in the *CA* affect the ALP and TH responses to stress in *Drosophila*, we calculated ALP and TH stress reactivities. No difference in ALP stress reactivity was found among the control groups (40±2 for *p[UAS-RNAi-InR]/CyO::arm-GFP*, 40±2 for *p[UAS-RNAi-InR]/CyO* and 37±3 for *Aug21-Gal4/CyO::arm-GFP*). A lack of differences is also observed for TH stress reactivity in these groups, with values of 58±1 for *p[UAS-RNAi-InR]/CyO::arm-GFP*, 63±1 for *p[UAS-RNAi-InR]/CyO* and 59±2 for *Aug21-Gal4/CyO::arm-GFP*. In contrast, ALP and TH stress reactivities in *Aug21-Gal4/p[UAS-RNAi-InR* females were significantly decreased: 19±1 for ALP and 45±1 for TH (one-way ANOVA ($F_{(3, 78)}$=31.48, $p < 0.001$) on ALP stress reactivity and ($F_{(3, 100)}$=67.58, $p < 0.00001$) on TH stress reactivity).

Juvenile hormone rescues alkaline phosphatase and tyrosine hydroxylase activities and their stress reactivities in females with decreased InR expression in CA

To determine whether the observed changes in the activity and stress reactivity of ALP and TH in *Aug21-Gal4/p[UAS-RNAi-InR]* females are related to the decreased JH levels in these animals, we studied the effect of JH dissolved in acetone on these parameters. For comparison, *Aug21-Gal4/p[UAS-RNAi-InR]* and *p[UAS-RNAi-InR]/CyO::arm-GFP* females were treated with pure acetone. Half of the individuals in each group under study were exposed to heat stress.

As shown in Fig 5B and D, JH treatment of *Aug21-Gal4/p[UAS-RNAi-InR]* (InR-) females increased ALP and TH activities to levels typical of *p[UAS-RNAi-InR]/CyO::arm-GFP* flies (InR+(1)). Comparison of JH treatment and heat stress effects on the ALP and TH activities of *Aug21-Gal4/p[UAS-RNAi-InR]* females by two-way ANOVA with JH treatment and temperature as fixed factors revealed significant effects of JH treatment ($F_{(1, 36)}$=158.43, $p < 0.00001$) and heat stress ($F_{(1, 36)}$=267.89, $p < 0.00001$) on ALP activity and of JH treatment ($F_{(1, 70)}$=57.85, $p < 0.00001$) and heat stress ($F_{(1, 70)}$=332.10, $p < 0.00001$) on TH activity. Interaction of these factors was found for both ALP ($F_{(1, 36)}$=82.41, $p < 0,00001$) and TH ($F_{(1, 70)}$=44.01, $p = 0.0038$). ALP and TH stress reactivities in JH-treated *Aug21-Gal4/p[UAS-RNAi-InR]* females (42±1 and 54±1, correspondingly) were also increased compared to acetone-treated *Aug21 Gal4/p[UAS-RNAi-InR]* females (18±4 and 36±1, correspondingly), and approached the levels of *p[UAS-RNAi-InR]/CyO::arm-GFP* females treated with acetone (39±1 for ALP and 56±1 for TH). The differences in stress reactivity between JH and acetone-treated *Aug21-Gal4/p[UAS-RNAi-InR]* females are significant (one-way ANOVA $F_{(1, 18)}$=41.33, $p < 0.0001$ on ALP stress reactivity and($F_{(1, 43)}$=121.71, $p < 0.001$ on TH stress reactivity).

Discussion

As mentioned above, InR expression was detected in *CA*, the gland that produces JH (Belgacem and Martin, 2006). Belgacem and Martin (2007) have also shown that InR in *CA* controls the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the enzyme that catalyses the synthesis of mevalonate, the precursor of the JH family (Belles et al., 2005)) in adult *Drosophila. In vitro*, it has been shown that a mutation of the *InR* gene leads to decreased JH production in adult *Drosophila* (Tatar et al., 2001, Tu et al., 2005).

To study the effect of RNAi knockdown of *InR* in the *CA* on JH synthesis *in vivo*, we used JH degradation level, stress reactivity of the JH metabolic system, and fecundity as indicators of JH production. Indeed, in *D. melanogaster* females, JH biosynthesis and degradation tend to show

an inverse relationship; JH production measured *in vitro* (Altaratz *et al*., 1991) is significantly higher in 1-day-old than in 5 to 6-day-old females (wild-type strain *Canton S*), whereas JH degradation measured *in vivo* is significantly lower in young than in mature *Canton S* females (Gruntenko *et al.*, 2003b). The dramatically decreased JH production measured *in vitro* in *D. melanogaster apterous*^{56f} mutant females (Altaratz *et al*., 1991) is paralleled by a marked increase in the measured *in vivo* activities of JH-esterase and JH-epoxide hydrolase, the enzymes that degrade JH (Gruntenko *et al.*, 2003b). In addition, we have demonstrated that a pharmacological increase in JH level in wild-type *D. virilis* females leads to a decrease in JH degradation (Rauschenbach *et al*., 2004). Terashima and Bownes (2005) found that JH treatment of *D. melanogaster* females resulted in decreased expression of the *JH-epoxide hydrolase 3* gene. More recently, we showed that decreased JH synthesis resulting from genetic ablation of a subset of *CA* cells leads to increased JH degradation and stress reactivity of the JH metabolic system in *D. melanogaster* females and to a dramatic decrease in their fecundity (Gruntenko *et al*., 2010). Taken together, these studies indicate that JH degradation, JH stress reactivity and fecundity may be useful parameters for assessing the level of JH synthesis *in vivo.* Assessment of these parameters in young female *D. melanogaster* provided evidence that RNAi-knockdown of *InR* in the *CA* leads to decreased JH production; in *Aug21-Gal4/p[UAS-RNAi-InR]* females, JH degradation and the stress reactivity of the JH metabolic system are increased (see Fig. 2), while fecundity is dramatically decreased (see Fig. 3A). The results of the experiments with JH treatment also indicate the decreased JH synthesis in these females: the exogenous hormone rescued JH degradation (see. Fig. 2B), JH stress reactivity and fecundity (see Fig. 3B) in the females *Aug21-Gal4/p[UAS-RNAi-InR]* to the levels of control flies.

In earlier work, we showed that JH regulates DA content in *Drosophila*: an increase or decrease in JH titer leads to a decrease or an increase in DA content *via* an increase or a decrease in DAT activity in young females (Rauschenbach *et al*., 2008, 2011; Gruntenko et al., 2012a). Considering this, we supposed that the IIS, by controlling the level of JH, could also affect the metabolism of DA. Measurement of DA content and DAT activity in *Aug21-Gal4/p[UAS-RNAi-InR]* females, which specifically downregulate InR in the *CA*, and in control *p[UAS-RNAi-InR]/CyO::arm-GFP* flies (see Fig. 4A,B) confirmed our supposition: DAT activity is decreased and DA level is increased in 1-day-old *Aug21-Gal4/p[UAS-RNAi-InR]* females. The fact that exogenous JH restored DAT activity (see Fig. 4C) indicates that the influence of the IIS on DA metabolism is indeed mediated by JH.

As we previously demonstrated, DA downregulates the activity of the enzymes of its synthesis, ALP and TH (Gruntenko et al., 2009; Bogomolova et al., 2010). Taking this into account, one might expect that the activities of ALP and TH would be changed in females with increased DA levels resulting from decreased InR expression in the *CA*. Measurements of the activities of these enzymes confirmed our expectations: in *Aug21-Gal4/p[UAS-RNAi-InR]* females, ALP and TH activities were decreased compared to control *p[UAS-RNAi-InR]/CyO::arm-GFP* females (see Fig. 5A,C). The effects of RNAi knockdown of *InR* in the *CA* on ALP and TH activities, as well as on DAT, are mediated by JH as shown by the fact that application of JH to *Aug21-Gal4/p[UAS-RNAi-InR]* flies restores the activity of both enzymes of DA synthesis (see Fig. 5B,D).

It is important to emphasise that decreased InR expression in the *CA* leads to defects in the development of the neuroendocrine stress reaction. In *Aug21-Gal4/p[UAS-RNAi-InR]* females, the response of all of the studied components of the stress reaction to heat treatment is changed. Note that the effects of decreased InR expression in the *CA* on the stress response are mediated by JH; exogenous JH restores stress reactivity of the JH and DA metabolic systems in the *Aug21-Gal4/p[UAS-RNAi-InR]* females to the level of that in control flies.

The results of our research provide evidence that IIS regulates DA metabolism indirectly *via* JH in females of *D. melanogaster* and show that it has an effect on the neuroendocrine stress reaction and that it interacts with JH in the control of reproductive functions in this species.

Materials and methods

Drosophila strains and genetic experiments

The following *Drosophila* strains were utilised in the current study: (1) the *Aug21-Gal4/CyO::arm-GFP* strain carrying the driver *Aug21-Gal4* that is expressed specifically in the *CA* of larvae (Mirth *et al*., 2005) and adults (Gruntenko et al., 2012a) of *D. melanogaster*; (2) the *p[UAS-RNAi-InR]/CyO* strain carrying an RNAi against the *InR* gene inserted on the chromosome II. Crossing *Aug21-Gal4/CyO::arm-GFP* with *p[UAS-RNAi-InR]/CyO* flies leads to two types of progeny: *Aug21-Gal4/p[UAS-RNAi-InR]*, where downregulation of InR is targeted to *CA* cells, and *p[UAS-RNAi-InR]/CyO::arm-GFP* siblings are used as control. The parental strains serve as additional controls. Note that the presence of mutations in the balancer chromosome CyO should not affect the results of the study as we have previously demonstrated that these mutations do not affect the parameters studied in this work (Gruntenko et al., 2010, 2012a,b). To permit

visualisation of *CA* cells, the *Aug21-Gal4::UAS-RFP* strain was produced by recombination of *Aug21-Gal4* with P{*UASp-Act5C.mRFP*} (the strain *w*;P{UASp-Act5C.mRFP}38* was obtained from the Bloomington Drosophila Stock Center).

Crosses were maintained at 29°C to enhance RNAi-induced phenotypes (Fortier and Belote, 2000; Draper et al., 2007) in a 12 h light:12 h dark (LD 12:12) cycle on standard *Drosophila* medium. After eclosion, flies were collected (flies eclosed within 3 to 4 hours were pooled) and maintained at 25°C.

Immunohistochemical analyses

Immunohistochemistry was performed as described by Gruntenko *et al*. (2012b). The cardia and *corpus cardiacum*/*corpus allatum* complex from flies selectively expressing red fluorescent protein (RFP) in *CA* cells were dissected and fixed in 4% paraformaldehyde (Sigma-Aldrich, USA), incubated 2 hours at room temperature and washed three times in PBS and three times in PBST (PBS + 0,05% Triton X-100). The tissues were subsequently blocked for three hours in 10% normal goat serum in PBST. A primary mouse monoclonal IgG1 anti-insulin receptor (α subunit) antibody (Millipore Corporation, Billerica, MA, USA) at a dilution of 1:20 was added, and the tissues were incubated overnight at 4°C. After three washes in PBST, bound primary antibodies were detected by a secondary antibody, ALEXA488 goat anti-mouse IgG (Invitrogen, Molecular Probes, Eugene, OR, USA), at a dilution of 1:500. After three washes in PBST and one in PBS, the tissues were embedded in Vectashield mounting medium for fluorescence (with DAPI, Vector Laboratories Inc., Burlingame, CA, USA) and observed by fluorescence microscopy (Axioscope 2, Zeiss, microscopy center, ICG).

JH-hydrolysing activity assay

JH hydrolysis was measured as described by Gruntenko *et al*. (2012a). Each fly was homogenised in 30 µl of ice-cold 0.1 M sodium phosphate buffer, pH 7.4, containing 0.5 mM phenylthiourea. The 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 unlabelled JH-III (Fluka, Buchs, Switzerland, additionally purified before use) and 12,500 dpm JH-III, [10-^{3}H(N)]- (15 Ci/mmol, Perkin Elmer, Waltham, MA, USA) 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 the organic and aqueous phases were placed in vials containing dioxane scintillation fluid and counted. Control

experiments showed a linear substrate-reaction product relationship; the activity measured is proportional to the amount of supernatant (i.e., enzyme concentration) (Gruntenko *et al.*, 2000). Before JH-hydrolysing activities were measured, half of the flies in each group under study were exposed to heat stress.

Heat stress

Flies were exposed to heat stress by transferring vials containing experimental flies from a 25°C incubator to a 38°C incubator for 1 hour (when examining DA and TH responses to stress) or for 1.5 hours (when examining ALP and JH degradation responses to stress).

JH treatment

Newly eclosed females were placed in vials with standard medium (five flies per vial). Twenty-four hours later, the females were treated with 0.1 μg JH-III (Sigma-Aldrich, USA) dissolved in 0.5 μl of acetone. Control females were treated with acetone (0.5 μl). Ten hours after JH or acetone application, the flies were frozen in liquid nitrogen and stored at -20°C. Half of the JH- and acetone-treated flies were exposed to heat stress.

Dopamine concentration measurements

DA concentration was measured as described by Gruntenko *et al*. (2012a). Flies were homogenised 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 syringe filter (Whatman, Schleicher&Schuell, 4 mm/0.45 μm, Germany), and 10 μl was injected directly onto an HPLC column through a valve fitted with a 20-μl sample loop. Chromatography was carried out in a C18 reverse-phase column (Diaspher-110-C18, 2.1x150 mm, 5 μm average particle size, BioChemMak, Russia) using an Agilent 1100 HPLC system with a quaternary pump, vacuum degasser and thermostatically controlled column compartment. The separated compounds were detected simultaneously by a variable wavelength detector (10-mm path length, 13-μl cell volume) set at 280 nm. Signals from the UV detector were recorded and integrated by a PC (Agilent ChemStation A.08.04). The flow rate was maintained at 0.3 ml/min. The mobile phase consisted of 0.025 M KH_2PO_4 (pH 3.0), 1 mM sodium octanesulfonate as an ion-pair reagent and 5% (v/v) acetonitrile. The concentration of DA was calculated by comparing the peak areas of the sample with those of standards. The identity of the DA peak in *Drosophila* samples was confirmed by comparison of its retention time with that of the standard mixture. The UV spectra of the DA peaks in *Drosophila* samples corresponded to the UV spectrum of DA. Moreover, we added excess quantities of DA (20 ng/100 μl of sample) to some *Drosophila* samples to register the changes in area of the appropriate peaks.

DA-dependent arylalkylamine N-acetyltransferase activity measurements

DAT activity was measured as described previously (Rauschenbach et al., 2008). Flies were homogenised on ice in 0.05 M Tris buffer (pH 7.2), one individual in 60 µl. The homogenates were centrifuged 5 min at 13,030 g. The components of the reaction mixture were added to a cuvette as follows: 50 µl of 0.05 M Tris (pH 7.2, Sigma-Aldrich, USA), 50 µl of acetyl CoA (0.5 mM, Fluka, Buchs, Switzerland, in 0.05 M Tris (7.2)), 25 µl of 12 mM phenylthiourea (Fluka, Buchs, Switzerland), 25 µl of substrate (40 mM DA, Sigma, Steinheim, Germany, in 0.001 N HCl), 50 µl of the supernatant, and 50 µl of 5,5-dithiobis-(2-nitro-benzoic acid) (2.4 mM, Fluka, Buchs, Switzerland, in 0.05 M Tris). The samples were incubated for 2 min at room temperature in the dark. The optical density of the reaction product was measured with a double-beam spectrophotometer (UV-2401PC, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 405 nm against the reaction zero point. DAT activity is represented in relative units (optical density x 100).

Alkaline phosphatase activity measurements

ALP assays were performed as described previously (Rauschenbach et al., 2007). Flies were homogenised on ice in 0.1 M Tris-phosphate buffer, pH 8.60 (Sigma-Aldrich, USA) (1 fly in 20 µl). The homogenates were centrifuged for 5 min at 13,030 g. Enzyme activity in the supernatant was determined using α-naphthylphosphate (ICN, Moscow, Russia) as substrate and fast blue RR salt (Chemapol, Czech Republic) as stain. After centrifugation, the supernatant was transferred to a microtube (1.5 ml; Eppendorf, Germany) to which 1 ml of reaction mixture (100 ml 0.1 M Tris buffer pH 8.60, 100 mg α-naphthylphosphate, 100 mg fast blue RR salt, 230 µl 10% MnCl, 230 µl 10% MgCl, 0.5 g polyvinylpyrrolidone (ICN, Moscow, Russia), and 2 g NaCl) was added. Incubation was carried out at room temperature in the dark for 25 min, and the reaction was stopped by the addition of 3 ml of ice-cold distilled water. The optical density of the obtained reaction product was measured at 470 nm using a Bio-Rad SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Philadelphia, PA, USA). ALP activity is represented in relative units (optical density x 100).

Tyrosine hydroxylase activity measurements

TH assays were performed as described previously (Gruntenko et al., 2009). Flies were homogenised in chilled 0.1 M acetate buffer, pH 7.0 (1 fly in 10 µl) and centrifuged at 13,030 g for 5 min; the supernatant was used for the reaction. The reaction mixture (100 µl) consisted of

0.1 mM L-tyrosine (Sigma, Steinheim, Germany), 1 μCi L-[ring-3,5-^{3}H] tyrosine (40 Ci/mmol, Perkin Elmer, Boston, MA, USA), 1 mM (6R)-5,6,7,8-tetrahydrobiopterin (Sigma-Aldrich, USA), 80 mM β-mercaptoethanol and 20 μg catalase (Fluka, Buchs, Switzerland) in 0.1 M acetate buffer (pH 7.0). Incubation was carried out for 15 min at 32°C. All assays were run in duplicate with zero time controls and terminated by addition of 1 ml of 7.5% activated charcoal (Darco G60, Fluka, Buchs, Switzerland). The assay mixtures were stirred for 10 min and centrifuged at 13,030 g for 5 min; 100 μl aliquots of the supernatant were transferred to scintillation vials and counted using a Rackbeta 1209 (Vellag, Turku, Finland) scintillation counter.

Fecundity analysis

Fecundity analysis was performed as follows: 3 newly eclosed females and 3 males were placed into vials, the bottom and 1 cm of the walls of which were covered with filter paper soaked in 0.5 ml of nutritional medium containing 0.5% sucrose and 0.1% yeast. The flies were transferred to vials with fresh medium daily. To study the effect of JH on fecundity, 3-day-old females were treated with acetone or JH as described above and their fecundity was evaluated. Fecundity was determined as the number of eggs laid by a female within 24 hours (the number of eggs laid in each vial was related to the number of females held in the vial for 24 hours).

Statistical analysis

The stress reactivities of DA, TH, ALP and JH were calculated as percent change in DA level or ALP, TH or JH-hydrolysing activities following heat stress relative to the values of the corresponding parameters obtained under normal conditions (each value obtained at 38ºC was compared to the average value obtained at 25ºC). The significance of differences between the data sets was tested by one-way and two-way ANOVA. The data on fecundity were analysed using two-way mixed-design ANOVA. Statistical comparisons between groups were performed using the Newman-Keuls post-hoc test.

List of symbols and abbreviations

JH - juvenile hormone

CA - corpus allatum

20E - 20-hydroxyecdysone

DA – dopamine

OA – octopamine

ALP - alkaline phosphatase

TH - tyrosine hydroxylase

DAT - DA-dependent arylalkylamine N-acetyltransferase

IIS - insulin/insulin-like growth factor signaling pathway

DILP - insulin-like peptide

InR – insulin-like receptor

Acknowledgements

We are grateful to Prof. Jean-Rene Martin (Laboratoire de Neurobiologie Cellulaire et Moleculaire, CNRS, Gif-sur-Yvette, France) for *D. melanogaster* strain *p[UAS-RNAi-InR]/CyO*. We are also grateful to Prof. Sheng Li (Institute of Plant Physiology and Ecology, Shanghai Institute of Biological Sciences of the Chinese Academy of Sciences) for *D. melanogaster* strain *Aug21-Gal4/CyO::arm-GFP*.

Competing interests

The author declares no competing financial interests.

Author contributions

All authors contributed to the conception, design, execution and interpretation of the findings.

Funding

The study was supported by the grants of Russian Foundation for Basic Research [12-04-00065, 13-04-00019] and Russian Federation Basic Project #VI.53.2.3.

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Figure legends

Fig. 1. InR immunoreactivity (green signal) in *corpus allatum* cells (red) of 1-day-old *D. melanogaster* females. (a) *CA* of *Aug21-Gal4;UAS-RFP* females; (a_1) the same gland in the green channel. (b) *CA* of *Aug21-Gal4/p[UAS-InR-RNAi];UAS-RFP* females; (b_1) the same gland in the green channel. Blue signal – cell nuclei stained with DAPI. Scale bar = 50 μm. (c) 1-day-old females: InR suppression - *Aug21-Gal4/p[UAS-InR-RNAi]* flies, control - *p[UAS-RNAi-InR]/CyO::arm-GFP* ones. (d) Weight of 1-day-old females.

Fig. 2. Effect of decreased InR expression in the *CA* on JH degradation. (A) JH degradation under normal conditions and upon heat stress (38ºC, 1.5 h) in *Aug21-Gal4/p[UAS-InR-RNAi]* females (InR-) with decreased InR expression in *CA* cells in comparison with control groups: *p[UAS-RNAi-InR]/CyO::arm-GFP* (InR+(1)), *p[UAS-RNAi-InR]/CyO* (InR+(2)), *Aug21-Gal4/CyO::arm-GFP* (InR+(3)). (B) The effect of JH and acetone treatment on JH degradation under normal conditions and upon heat stress (38ºC, 1.5 h) in InR- and InR+(1) females. The data are given as mean ± SE. The diamond indicates significant differences from InR- females; the asterisk indicates significant differences between heat-treated and control flies of the same genotype (Newman-Keuls post-hoc).

Fig. 3. Effect of decreased InR expression in the *CA* on reproduction. (A) The fecundity of *D. melanogaster* females with RNAi-knockdown of *InR* in *CA* (*Aug21-Gal4/p[UAS-InR-RNAi]*) *vs* control flies (*p[UAS-RNAi-InR]/CyO::arm-GFP*, *p[UAS-RNAi-InR]/CyO* and *Aug21-Gal4/CyO::arm-GFP*). (B) The effect of JH and acetone treatment on the fecundity of *Aug21-Gal4/p[UAS-InR-RNAi]* females. The time of JH or acetone application is shown with an arrow. Data are given as mean ± SE.

Fig. 4. Effect of decreased InR expression in the *CA* on DA content and degradation. (A) DA level under normal conditions and upon heat stress (38ºC, 1 h) in *Aug21-Gal4/p[UAS-InR-RNAi]* females (InR-) with decreased InR expression in *CA* cells in comparison with *p[UAS-RNAi-InR]/CyO::arm-GFP* (InR+(1)) females. (B) DAT activity under normal conditions in InR- and InR+(1) females. (C) The effect of JH and acetone treatment on DAT activity in InR- and InR+(1) females. Data are given as mean±SE. The diamond sign indicates the significant differences from InR- females, and the asterisk indicates significant differences between heat-treated and control flies of the same genotype (Newman-Keuls post-hoc).

Fig. 5. Effect of decreased InR expression in the *CA* on DA metabolism. (A) ALP activity under normal conditions and upon heat stress (38°C, 1.5 h) in *Aug21-Gal4/p[UAS-InR-RNAi]* females (InR-) with decreased InR expression in *CA* cells in comparison with control groups: *p[UAS-RNAi-InR]/CyO::arm-GFP* (InR+(1)), *p[UAS-RNAi-InR]/CyO* (InR+(2)), *Aug21-Gal4/CyO::arm-GFP* (InR+(3)). (B) The effect of JH and acetone treatment on ALP activity under normal conditions and upon heat stress (38°C, 1.5 h) in InR- and InR+(1) females. (C) TH activity under normal conditions and upon heat stress (38°C, 1 h) in InR-, InR+(1), InR+(2) and InR+(3) females. (D) The effect of JH and acetone treatment on TH activity under normal conditions and upon heat stress (38°C, 1 h) in InR- and InR+(1) females. Data are given as mean ± SE. The diamond sign indicates a significant difference from InR- females, and the asterisk indicates a significant difference between heat-treated and control flies of the same genotype (Newman-Keuls post-hoc).

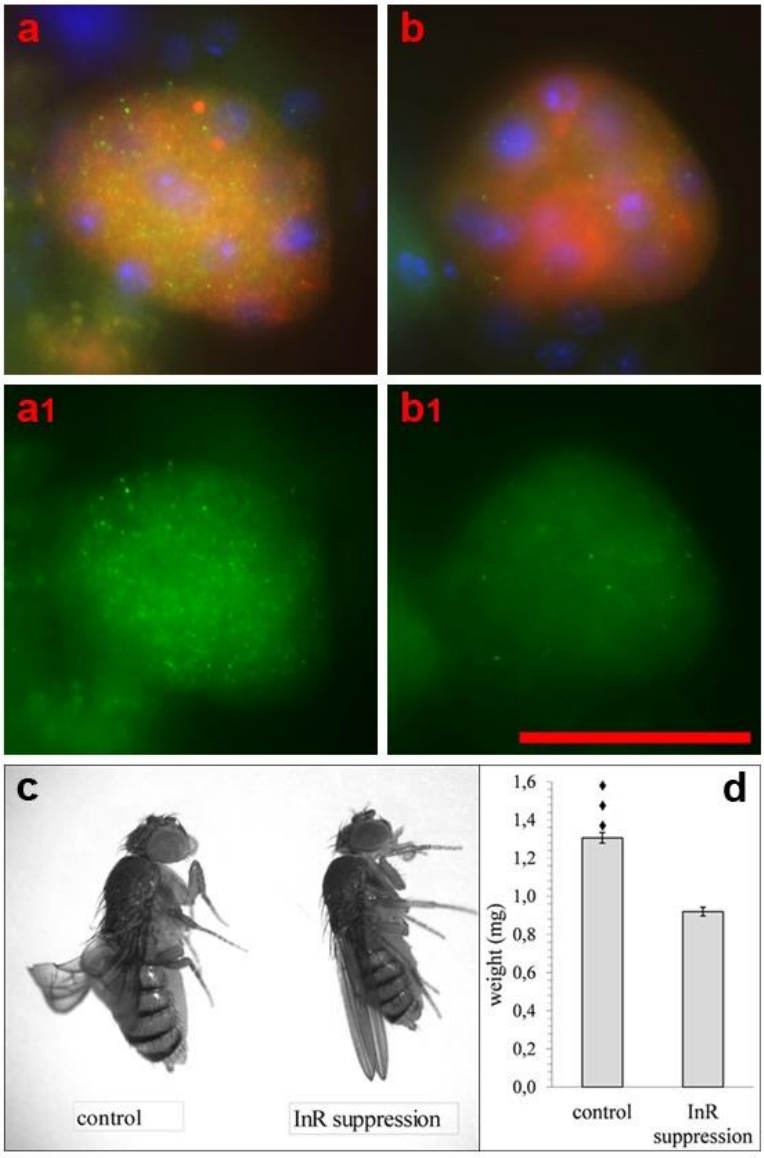

Fig. 1.

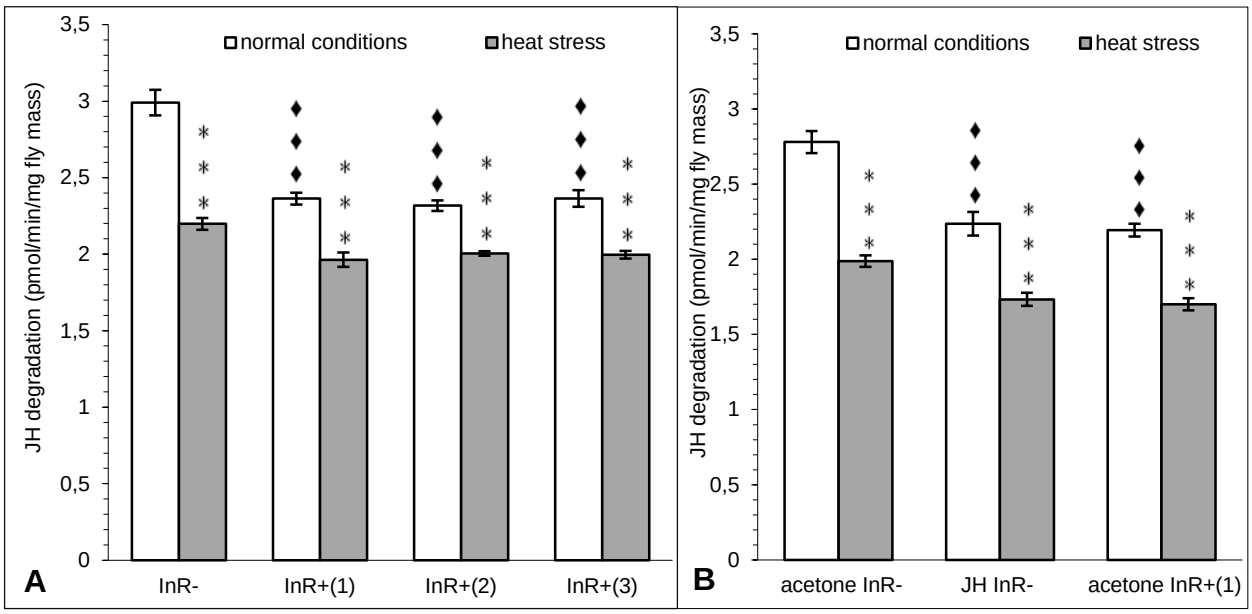

Fig. 2.

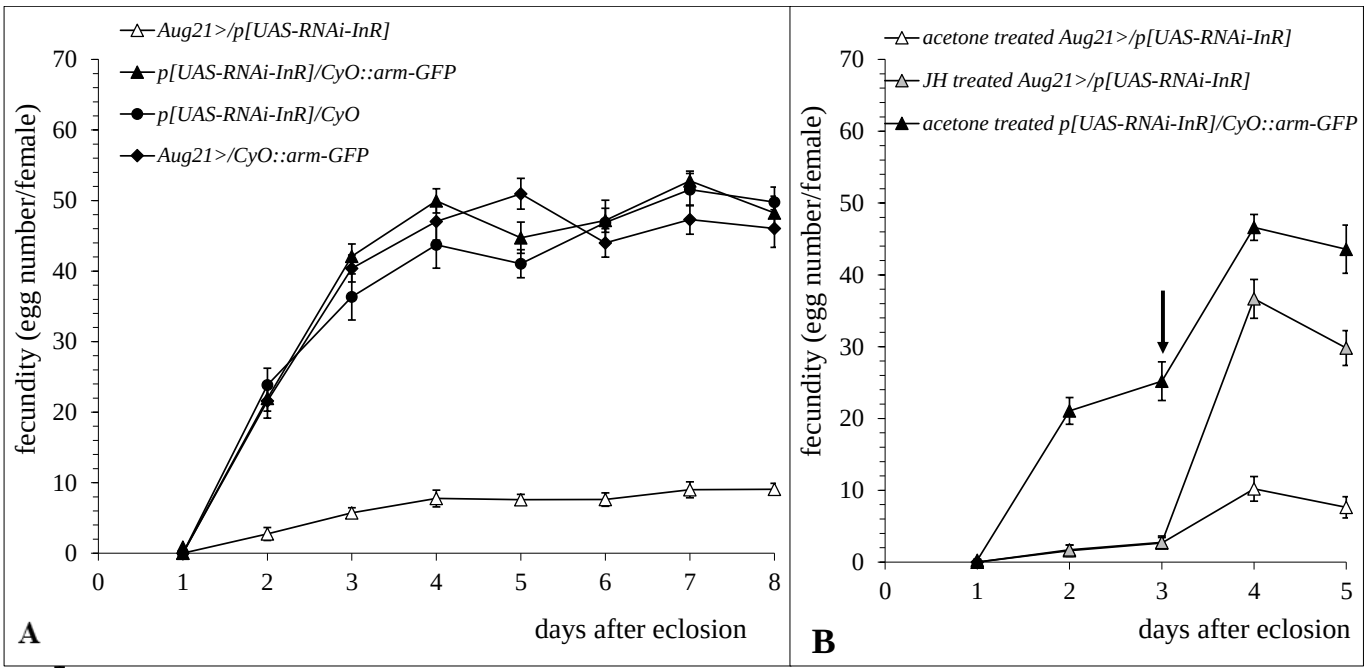

Fig. 3.

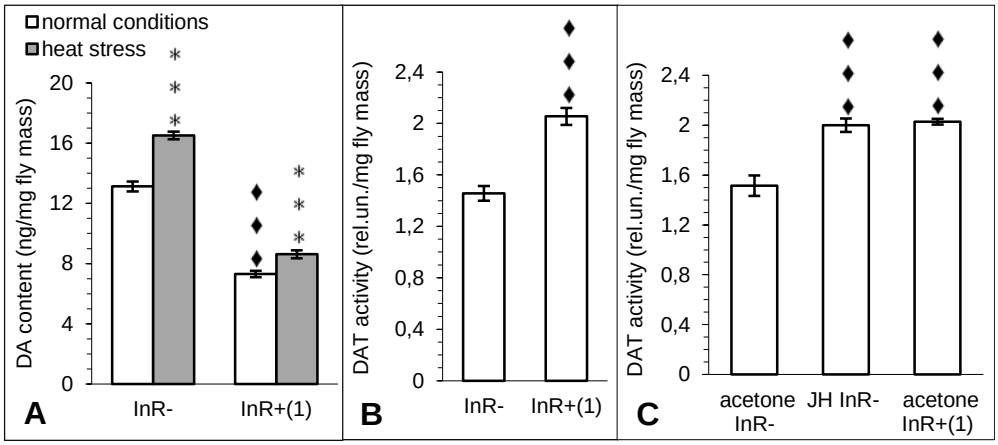

Fig. 4.

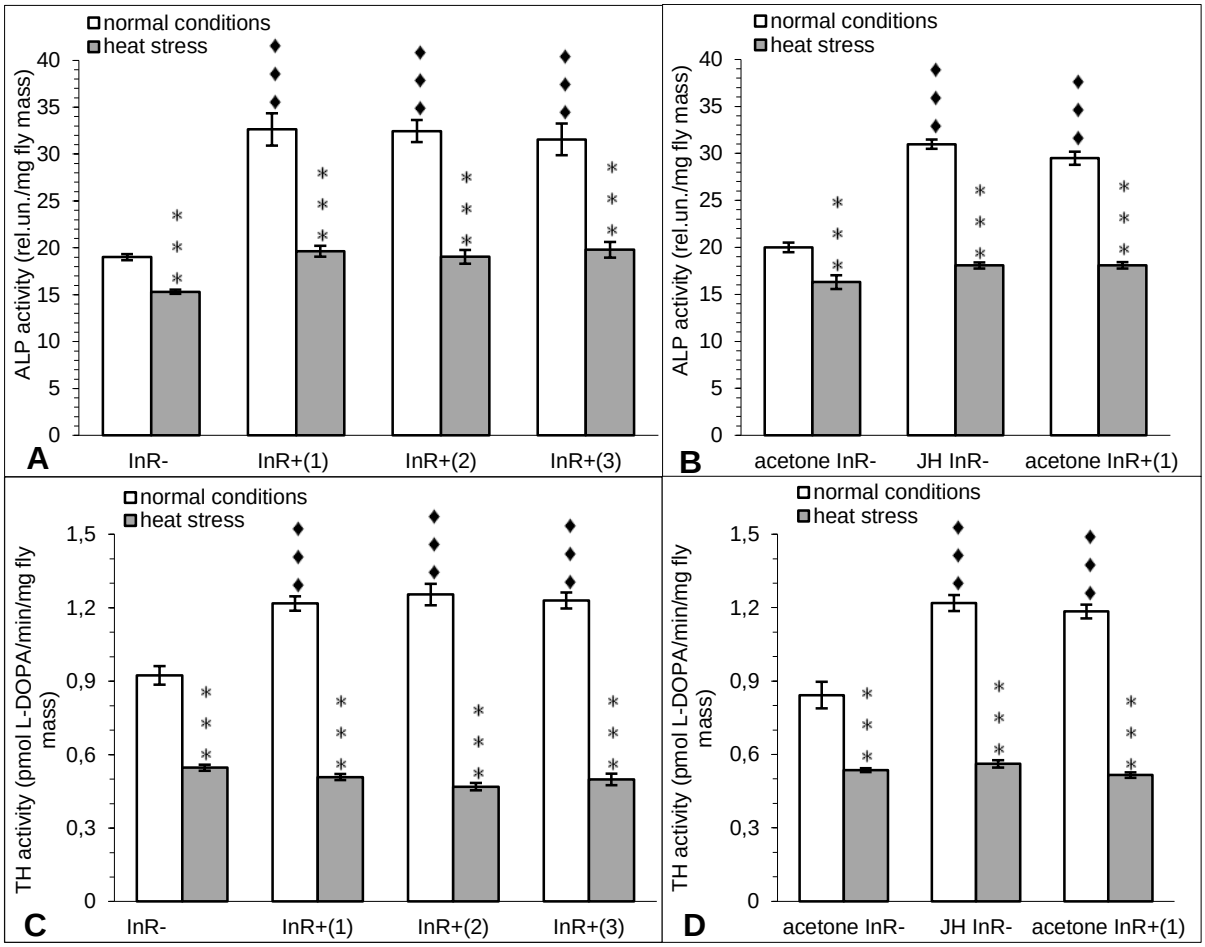

Fig. 5.