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Regulation of *insulin-like peptide* expression in adult *Blattella germanica* females

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ABSTRACT

The insulin-IGF-signalling (IIS) pathway regulates key processes in metazoans. The pathway is activated through the binding of the ligands, which in insects are usually referred to as insulin-like peptides (ILPs), to a class of receptor tyrosine kinases, the insect insulin receptor. To study the pathway regulation, it is therefore essential to understand how ILPs are produced and released. In this study we analysed the factors that regulate the expression of the seven *ILPs* (*BgILPs*) expressed in adult females of the German cockroach, *Blattella germanica*. The results showed that the starvation-induced expression reduction of brain *BgILP3*, *5* and *6* and fat body *BgILP7* is not due to reduced juvenile hormone (JH) or decreased TOR pathway activity. In addition, depletion of *FoxO* in starved females did not correct the low levels of these *BgILPs*, but even reduced further *BgILP5* expression, indicating the need to maintain certain basal levels of *BgILP5* even during starvation. Furthermore, JH promoted increased *BgILP5* and decreased *BgILP3* expression in the brain, an effect that required Methoprene-tolerant (Met), the JH receptor, but not Krüppel homolog 1 (Kr-h1), the main JH transducer. On the other hand, JH inhibited the expression of *BgILP7* in the fat body, although in this case, the action required both Met and Kr-h1. In addition, JH reduction treatments produced a decrease in the expression of the *insulin receptor* in the fat body, which suggests an increase in IIS. The results show a peculiar regulation of *ILP* expression in adult *B. germanica* females, which is clearly different than that seen in other species. This is understandable given that gene duplications in recent clades have resulted in different sets of *ILP* genes, involving substantial changes in gene regulatory networks.

1. Introduction

For an organism to function correctly, the cells of the various tissues and organs must communicate with each other. In response to a stimulus, the endocrine system enables that information, in the form of a chemical messenger or hormone, to simultaneously reach the entire organism. Hormones can also act in a paracrine or autocrine manner, depending on how locally they act.

In the above sense, one of the most important hormonal systems is the insulin-IGF-signalling (IIS) pathway, which is well conserved in metazoans, including insects and vertebrates. In insects, IIS regulates key processes, such as cellular proliferation, growth, longevity, metabolism, reproduction, and caste determination (Claeys et al., 2002; Wu and Brown, 2006). In the German cockroach, *Blattella germanica*, IIS is involved in regulating growth, reproduction and metabolism (Abrisqueta et al., 2014, 2017; Süren-Castillo et al., 2012, 2014).

Thus, in *B. germanica*, RNAi-triggered depletion of one of the insulin receptors (InR Cluster I according to Smýkal et al., 2020), results in reduced nymphal growth (Abrisqueta et al., 2014). The RNAi of *InR* and *ribosomal S6 protein kinase* (*S6K*) determines an increase in the duration of the last nymphal instar, together with reduced juvenile hormone (JH) and vitellogenin (Vg) production (Abrisqueta et al., 2014, 2017).

The transcription factor FoxO is the main mediator in the IIS pathway (Greer and Brunet, 2005). When the pathway is activated, a phosphorylation cascade of different kinases ends with the phosphorylation of FoxO, causing it to be exported from the nucleus and, therefore, inactivated (Greer and Brunet, 2005; Kramer et al., 2003; Puig et al., 2003). When the pathway is inactive, dephosphorylated FoxO moves into the nucleus and activates the transcription of *InR*, *hypertrehalosemic hormone*, *Brummer lipase*, or *glycogen phosphorylase*, as observed in *B. germanica* (Abrisqueta et al., 2014; Süren-Castillo et al., 2014), or inhibits *Vg* transcription in both *B. germanica* (Süren-Castillo et al., 2012) and the beetle *Tribolium castaneum* (Sheng et al., 2011).

In adult *B. germanica* females, seven ILPs have been described. Six of

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these (*BgILP1*, *2*, *3*, *4*, *5,* and *6*) are mainly expressed in the brain, whereas the fat body expresses *BgILP7,* and the ovaries also express *BgILP2* (Castro-Arnau et al., 2019). Due to the difficulty of identifying orthologies between these genes and those described in other insects (Antonova et al., 2012), the numbering of those found in *B. germanica* simply corresponds to the order in which they were discovered. Recently, Veenstra (2020) reported the occurrence of a *BgILP8*, which was found to be abundantly expressed just in transcriptomes of the male reproductive organs and fat body. Although it was extensively tested, we were unable to identify the roles of BgILPs in vitellogenesis or reproduction of adult females (Castro-Arnau et al., 2019). However, we determined that some *BgILPs* are differentially expressed when starvation and feeding conditions are compared, and a reciprocal compensatory effect in the expression of *BgILP3* and *BgILP5* has also been reported (Castro-Arnau et al., 2019).

Studying the regulation of *ILP* expression will help us to understand how IIS pathway activity is regulated. In this work we scrutinised the factors that regulate the expression of *B. germanica ILPs*, in particular during starvation and in response to the action of JH. The results show that various factors regulate *BgILP* expression, some of which are similar to those described for other species, while others act differently. This points to the difficulty of generalising *ILP* regulation mechanisms in insects.

2. Material and methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark on dog food and water at 29 ± 1 °C and 60–70% relative humidity. For the starvation assays, animals received only water after the imaginal molt. Dissections were carried out in Ringer's saline on carbon dioxide-anesthetized specimens. After dissection, tissues were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA extraction, cDNA synthesis and quantitative real-time PCR analysis

Total RNA was extracted using the GenElute™ Mammalian total RNA (Sigma) or HigherPurity™ Tissue Total RNA Purification (canvax) kits. cDNAs were synthetized from total RNA as previously described (Montañés et al., 2021). In the case of fat body and ovary, 1 μg of total RNA was used, whereas in the case of brain and CC-CA, we lyophilized and concentrated the sample to use the total amount of the RNA. The absence of genomic contamination was confirmed using a control without reverse transcription. Quantitative real-time PCR analyses were carried out as previously described (Ons et al., 2015). Primer sequences to amplify the different *BgILPs*, *FoxO*, *TOR*, *Vg*, *JHAMT, Met*, *Kr-h1*, *InR*, *Actin 5C* and *eukaryotic initiation factor 4a* (*EIF4a*) have been reported elsewhere (Abrisqueta et al., 2014; Castro-Arnau et al., 2019; Dominguez and Maestro, 2018; Irles and Piulachs, 2014; Lozano and Belles, 2011, 2014; Maestro et al., 2009; Süren-Castillo et al., 2012). In the analysis of fat body expression from the RNAi experiments for *TOR, JHAMT* and *Met*, *EIF4a* was used as the reference gene. In all the other cases, the reference gene was *Actin 5C*. The total reaction volume was 20 μL. All reactions were run in duplicate or triplicate.

2.3. RNA interference

dsRNAs were synthetized using MEGAscript™ RNAi kit (Invitrogen). To avoid the possible effects of protein depletion during nymphal development we injected 2 μl of dsRNA at a concentration of 1 μg/μl into the abdomen females on the first day of oothecal transport, using a 5 μl Hamilton® 75N syringe. Treatment was repeated the treatment on day 7. On the 12, oothecae were manually removed to synchronize the start of the second gonadotrophic cycle for all the experimental animals. The animals were dissected on day 5 of the second gonadotrophic cycle, which is, in all aspects, comparable to the first cycle. The primers used to generate the dsRNAs for *FoxO*, *TOR*, *JHAMT*, *Met* and *Kr-h1* are described elsewhere (Dominguez and Maestro, 2018; Lozano and Belles, 2011, 2014; Maestro et al., 2009; Süren-Castillo et al., 2012). A heterologous 441 bp fragment from the gene sequence of the *polyhedrin* of *Autographa californica* nucleopolyhedrovirus was used as negative control.

2.4. Juvenile hormone treatment

JH treatment was performed by topical application (Ons et al., 2015). Four days after the adult molt, in the case of the starvation experiment, or after removing the ootheca and triggering the second gonadotrophic cycle, in the case of the *JHAMT* RNAi experiment, the wings of the animals were cut and 1 μl of JH III (Sigma) diluted in analytical grade acetone at a concentration of 2 μg/μl or 20 μg/μl was topically applied on the abdominal tergites using a 5 μl Hamilton® 75N syringe. Controls were equivalently treated with acetone.

2.5. Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using IBM SPSS Statistics 24. In the case of experiments which included JH treatments, ANOVA with Tukey's test was performed. For the remaining experiments, comparison of results from control and treated animals was performed using Student's *t*-test.

3. Results

3.1. Regulation of ILP expression in starved females

Our first experiment was aimed at checking whether the reduced expression of brain *BgILP3*, *BgILP5* and *BgILP6*, fat body *BgILP7*, and the increased expression of ovarian *BgILP2* that was observed in starved compared to fed adult *B. germanica* females (Castro-Arnau et al., 2019), was due to the reduced JH production observed during starvation (Maestro et al., 2009). Thus, we treated 4-day-old starved females with 2 μg of either JH III or acetone, and quantified *BgILP* mRNA levels 24 h later. The results showed that *BgILP3*, *5*, *6* and *7* showed reduced mRNA levels in starved animals, although in the case of *BgILP6*, starvation produced a 48% reduction that didn't show significant differences compared to the fed animals in the statistical test. In the case of the ovary, starvation induced a 2.3-fold increase in *BgILP2* mRNA levels, although again this result didn't show significant differences compared to the fed animals in the statistical test. In addition, the treatment with JH did not reverse the starvation effect on *BgILP* expression (Fig. 1), although it did trigger a 9-fold increase in *vitellogenin* (*Vg*) expression in the fat body (Fig. S1).

One of the potential candidates involved in regulating the expression of *ILPs* during starvation is the transcription factor FoxO. For this reason, we determined the effect of *FoxO* depletion on *ILP* expression in starved females. To avoid possible effects of *FoxO* depletion on growth and development, we treated *B. germanica* adult females in the first day of ootheca transport with 2 μg of dsRNA targeting *FoxO* (ds*FoxO*). The treatment was repeated seven days later. We manually removed the ootheca on the twelfth day and kept the animals starved for five days, when we dissected them. The treatment produced a 48%, 78% and 46% reduction of *FoxO* mRNA levels in brain, fat body and ovary, respectively (Fig. S2). *FoxO* depletion produced a significant change in brain *BgILP5* mRNA. However, this change did not involve an increase, but rather an even greater reduction of *BgILP5* mRNA levels (Fig. 2). No significant changes were observed in the levels of brain *BgILP3* and *BgILP6*, fat body *BgILP7*, and ovarian *BgILP2* mRNA (Fig. 2 and Fig. S2).

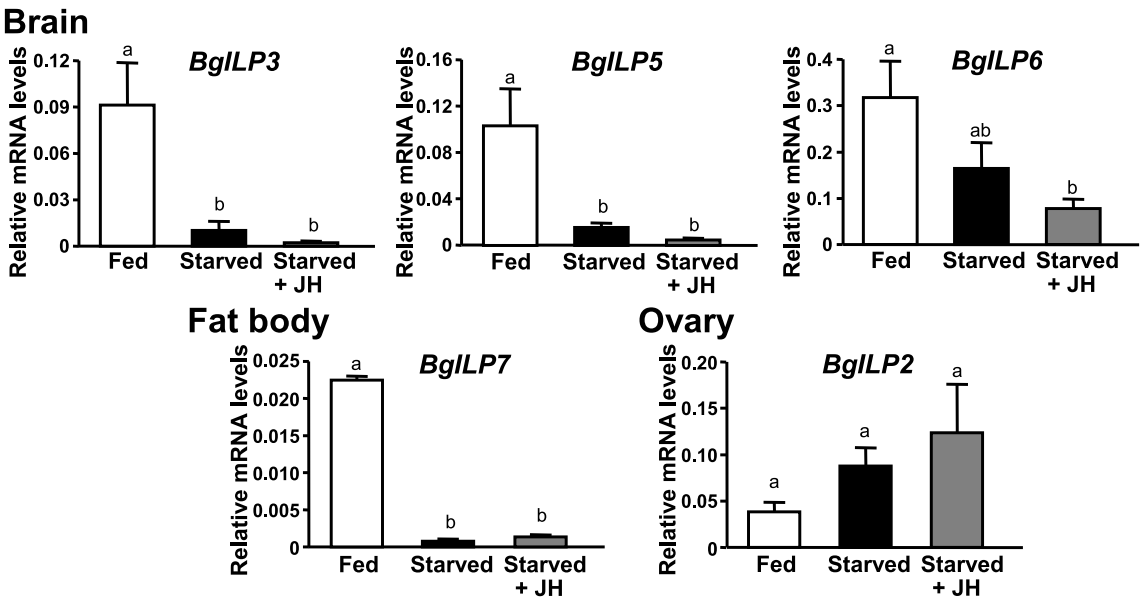

Fig. 1. *BgILPs* expression in fed, starved and starved treated with JH *B. germanica* females. Starved animals were treated 24 h before the dissections with 2 μg JH (Starved + JH) or acetone (Starved). Graphs show *BgILP3*, *5* and 6 mRNA levels in brains, *BgILP7* mRNA levels in fat bodies and *BgILP2* mRNA levels in ovaries from 5-day-old adult females. *Y*-axes indicate copies per copy of *Actin 5C*. The results are expressed as the mean ± S.E. (n = 3–4). The different letters (a–b) indicate groups with significant differences according to the ANOVA test (Tukey, $p < 0.05$).

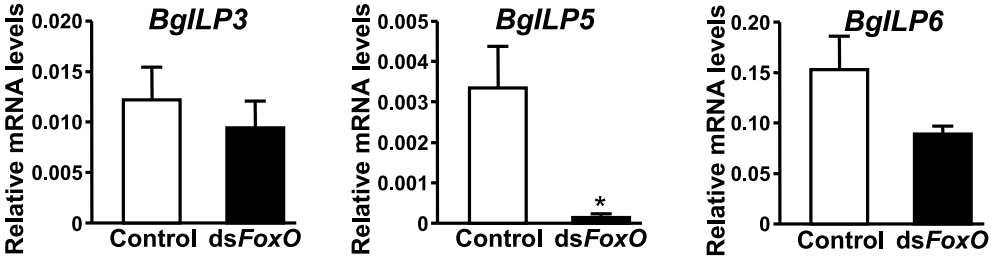

Fig. 2. Effect of *FoxO* RNAi on brain *BgILPs* expression of starved females. Adult *B. germanica* females were treated with dsRNA targeting *FoxO* (ds*FoxO*) or a heterologous dsRNA (Control) during the oothecal transport period, and dissections were performed on day 5 of the second gonadotrophic cycle (see Material and Methods). The animals were starved from the beginning of the second gonadotrophic cycle. Graphs show *BgILP3, 5 and* 6 mRNA levels in brains. *Y*-axes indicate copies per copy of *Actin 5C*. The results are expressed as the mean ± S.E (n = 4–5). Asterisks represent significant differences between Control and ds*FoxO* animals (Student's *t*-test, $^*p < 0.05$).

3.2. Effect of dsFoxO and dsTOR treatments in fed females

We then decided to analyse the effect of *FoxO* silencing on the expression of brain *BgILP5* (and the other brain *ILPs*) in fed females. We followed the same experimental protocol but, in this case, we allowed access to food after ootheca removal. The treatment produced a 49% reduction of brain *FoxO* mRNA levels (Fig. S3), but no significant expression changes were observed in any of the brain *ILPs*, although *BgILP5* mRNA levels showed a tendency to reduce (51% reduction on average) (Fig. S3).

Provided that the target of rapamycin (TOR) pathway is activated by circulating amino acids (Hansen et al., 2004; Kim and Guan, 2019), starvation also produces a reduction in TOR pathway activity. One possibility is, then, that the reduced expression of *BgILPs* was due to reduced TOR pathway activity. To investigate this possibility, we followed the same experimental protocol as in the previous experiment, this time treating with 2 μg of dsRNA targeting TOR (ds*TOR*) and providing food for the second gonadotrophic cycle. Dissections were once again performed five days after oothecal removal, at the peak vitellogenic period for control animals. The treatment resulted in 83%, 92% and 49% reduction of TOR mRNA levels in brain, fat body and ovary, respectively (Fig. S4). The results showed that *TOR* depletion did not produce the same effect as starvation in terms of *ILP* expression. In the brain, the treatment elicited an increase in *BgILP3* mRNA levels and a tendency towards reduced *BgILP5* mRNA levels (Fig. 3). In the fat body, ds*TOR* treatment produced a 4.3-fold increase in *BgILP7* mRNA levels (Fig. 3). Also in the case of the ovary, ds*TOR* treatment induced a 1.9-fold increase in *BgILP2* mRNA levels (Fig. 3).

3.3. Regulation of ILP expression by juvenile hormone

One of the effects of ds*TOR* treatment in *B. germanica* females is reduced juvenile hormone (JH) synthesis (Maestro et al., 2009). There is, therefore, the possibility that the effects on ILP expression observed after the ds*TOR* treatment were due to reduced levels of JH. Thus, we analysed the role JH plays in regulating *ILP* expression. To do this, we depleted the expression of *juvenile hormone acid O-methyltransferase* (*JHAMT*) (Fig. S5) by injecting dsRNA targeting *JHAMT* (ds*JHAMT*) using a similar protocol as in the previous treatments. The results showed that RNAi-triggered *JHAMT* depletion (Fig. S5) elicited an 88% decrease in brain *BgILP5* mRNA. The treatment also resulted in a tendency to increased *BgILP3* mRNA levels, although the differences with the controls were not statistically significant. The expression of the other brain *ILPs* was practically unaffected after *JHAMT* depletion (Fig. 4). To ascertain whether the observed effects on ILP expression were really due to the reduced JH levels, we repeated the RNAi treatment, treating the ds*JHAMT* females with 2 μg of JH III or acetone 24 h before the dissections. The results showed that JH treatment corrected the effects of

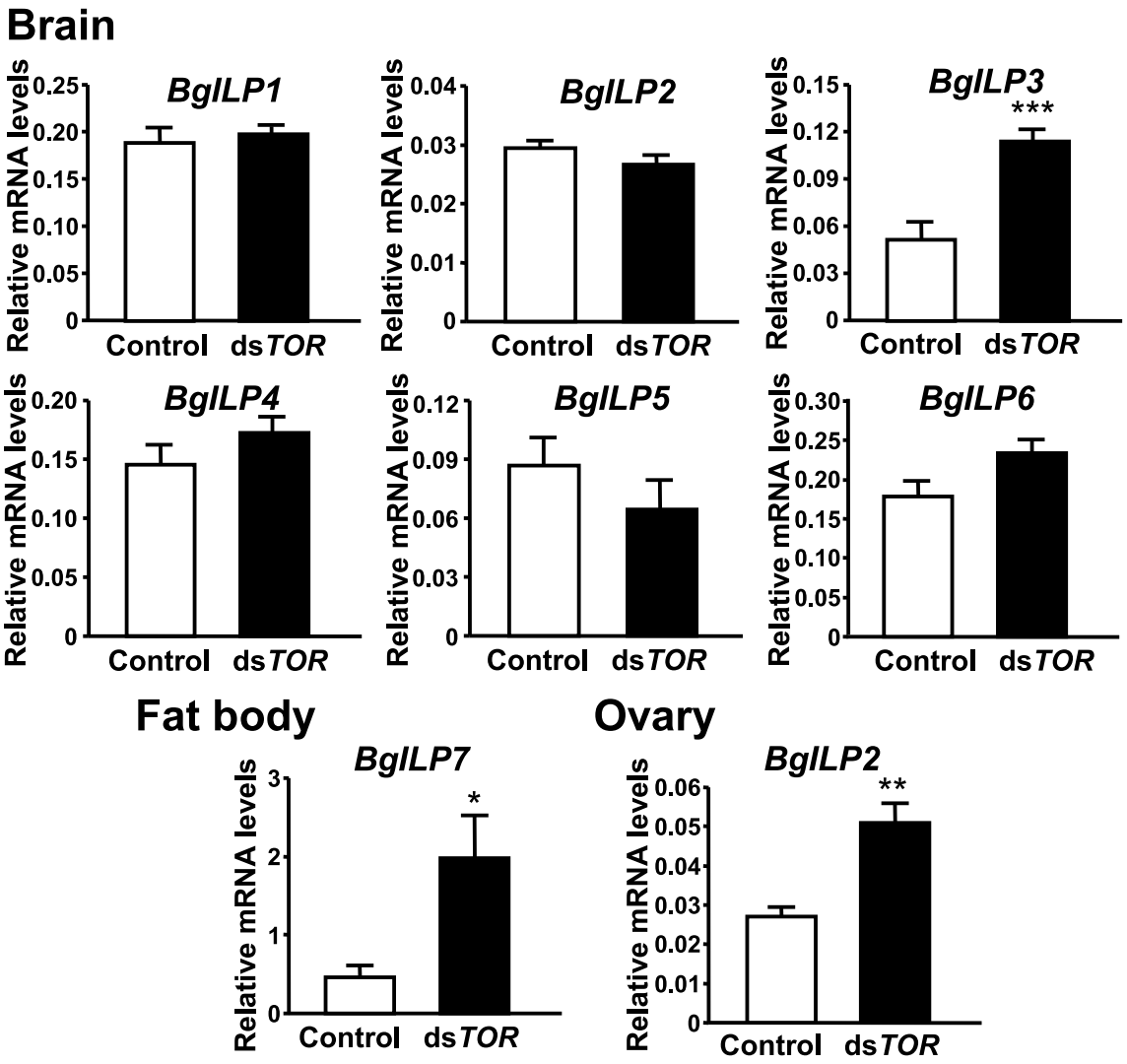

Fig. 3. Effect of *TOR* RNAi on brain, fat body and ovary *BgILPs* expression. Adult *B. germanica* females were treated with dsRNA targeting *TOR* (ds*TOR*) or a heterologous dsRNA (Control) during the oothecal transport period, and dissections were performed on day 5 of the second gonadotrophic cycle (see Material and Methods). Graphs show brain *BgILP1-6*, fat body *BgILP7* and ovary *BgILP2* mRNA levels. *Y*-axes indicate copies per copy of *Actin 5C* in the case of brains and ovaries and copies per copy of *EIF4a* in the case of fat bodies. The results are expressed as the mean ± S.E (n = 7–8 for brains, n = 5 for fat bodies and n = 3–4 for ovaries). Asterisks represent significant differences between Control and ds*TOR* animals (Student's *t*-test, $*p < 0.05$; $**p < 0.01$; $***p < 0.0005$).

the JH depletion on brain *ILPs*, reducing *BgILP3* mRNA levels and increasing *BgILP5* mRNA levels (Fig. 4).

In the ovary, ds*JHAMT* treatment produced a 3.7-fold increase in the expression of ovarian *BgILP2*, whereas the JH treatment reverted this increase, and elicited a slight increase of the basal follicle length (Fig. S5).

In the fat body, ds*JHAMT* treatment induced a 27-fold increase in *BgILP7* mRNA levels (Fig. 4). In this tissue, the 2 μg JH treatment did not correct the effect of the ds*JHAMT* treatment (results not shown). For this reason, we decided to repeat the experiment using a JH dose of 20 μg; this fully corrected the effect of the ds*JHAMT* treatment (Fig. 4).

3.4. Mechanism of action of JH in the regulation of ILPs

To determine the molecular mechanism through which JH regulates *BgILP* expression, we analysed the effects of RNAi-triggered depletion of *Met* (ds*Met*) and *Kr-h1* (ds*Kr-h1*) on *BgILP* mRNA levels. We used the same dsRNA treatment protocol as in the previous experiments. The ds*Met* treatment elicited a 50% reduction in brain *Met* mRNA levels and a 30% reduction in *Kr-h1* mRNA levels (Fig. 5). In addition, *BgILP5* expression showed an 82% reduction, whereas for *BgILP3* there was a tendency to increase (56% increase as average), although differences with respect to the controls were not statistically significant (Fig. 5). In ds*Kr-h1*-treated insects, although the treatment elicited a 52% reduction in *Kr-h1* mRNA levels (Fig. 5), we observed no differences in the levels of either *BgILP5* or *BgILP3* (Fig. 5). In addition, there were no changes in any of the other brain *ILP* (*BgILP1*, *2*, *4* and *6*) mRNA levels after the ds*Met* or ds*Kr-h1* treatments (Fig. S6).

In terms of the fat body, both ds*Met* and ds*Kr-h1* treatments elicited increased *BgILP7* expression, similar to that produced by the ds*JHAMT* treatment (Fig. 5), even though the 33% reduction of *Kr-h1* mRNA levels triggered by the ds*Kr-h1* treatment was not statistically significant. In addition, the ds*Met* treatment reduced Vg expression, although the ds*Kr-h1* treatment did not (Fig. 5).

In the ovaries, ds*Met* animals did not show a decrease in *Met* mRNA, although they showed a significant reduction in *Kr-h1* mRNA levels (Fig. S6). In addition, ds*Met* treatment mimicked the increase in *BgILP2* mRNA levels triggered by the ds*JHAMT* treatment but ds*Kr-h1* did not (Fig. S6).

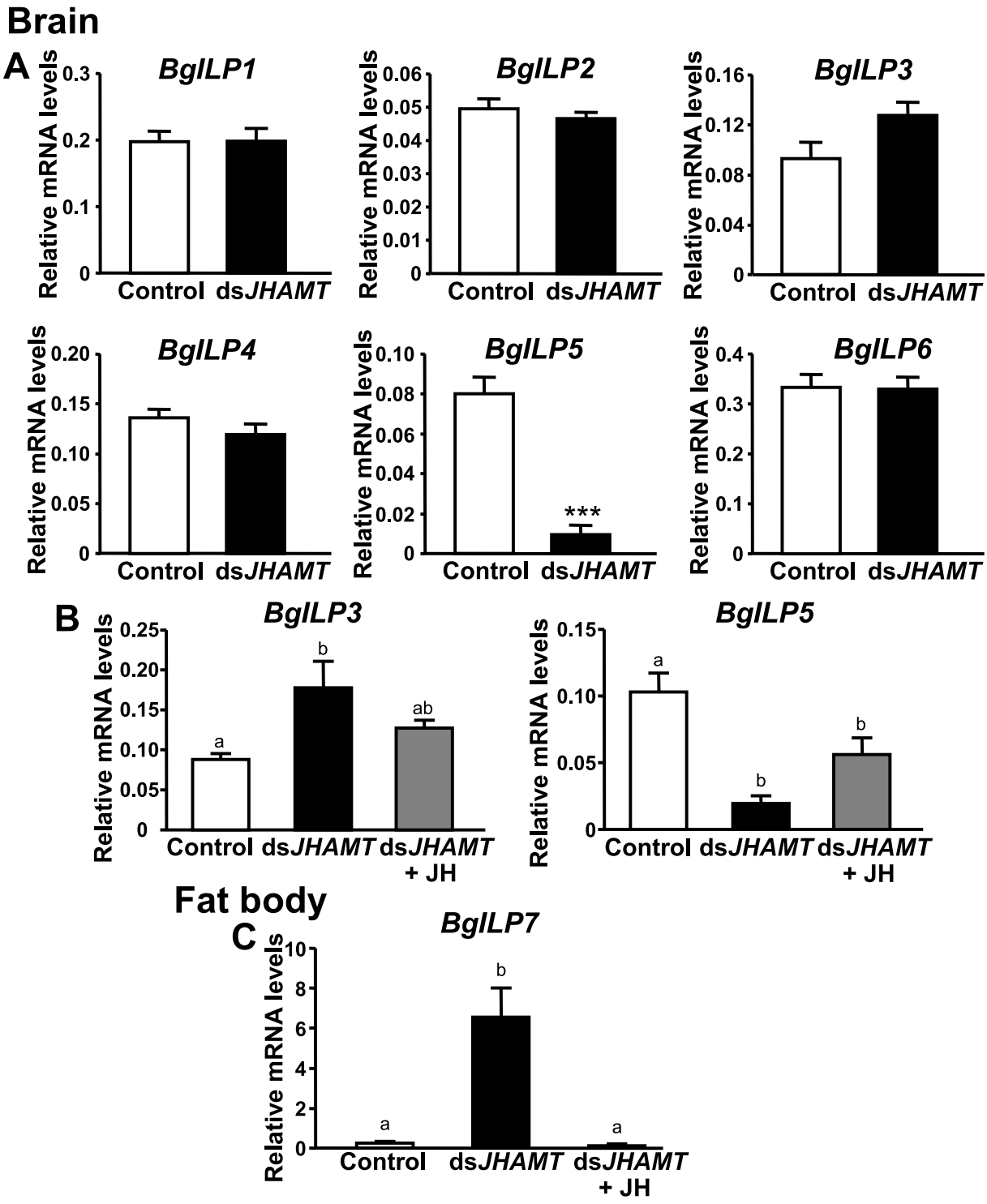

Fig. 4. Effect of *JHAMT* RNAi and JH treatment on brain and fat body *BgILPs* expression. Adult *B. germanica* females were treated with dsRNA targeting *JHAMT* (ds*JHAMT*) or a heterologous dsRNA (Control) during the oothecal transport period, and dissections were performed on day 5 of the second gonadotrophic cycle (see Material and Methods). For A, only Control and ds*JHAMT* treatment was performed (n = 9–10 for brains and n = 8–10 for fat bodies). For B and C, animals were treated 24 h before the dissections with JH: 2 μg in the case of brains and 20 μg in the case of fat bodies (ds*JHAMT* + JH), or with acetone (Control and ds*JHAMT*). (n = 5 for brains and n = 4 for fat bodies). Graphs show brain *BgILP1-6* and fat body *BgILP7* mRNA levels. *Y*-axes indicate copies per copy of *Actin 5C* in the case of brains and copies per copy of *EIF4a* in the case of fat bodies. The results are expressed as the mean ± S.E. In A, asterisks represent significant differences between Control and ds*JHAMT* animals (Student's *t*-test, ***$p < 0.0001$). In B and C, the different letters (a–b) indicate groups with significant differences according to the ANOVA test (Tukey, $p < 0.05$).

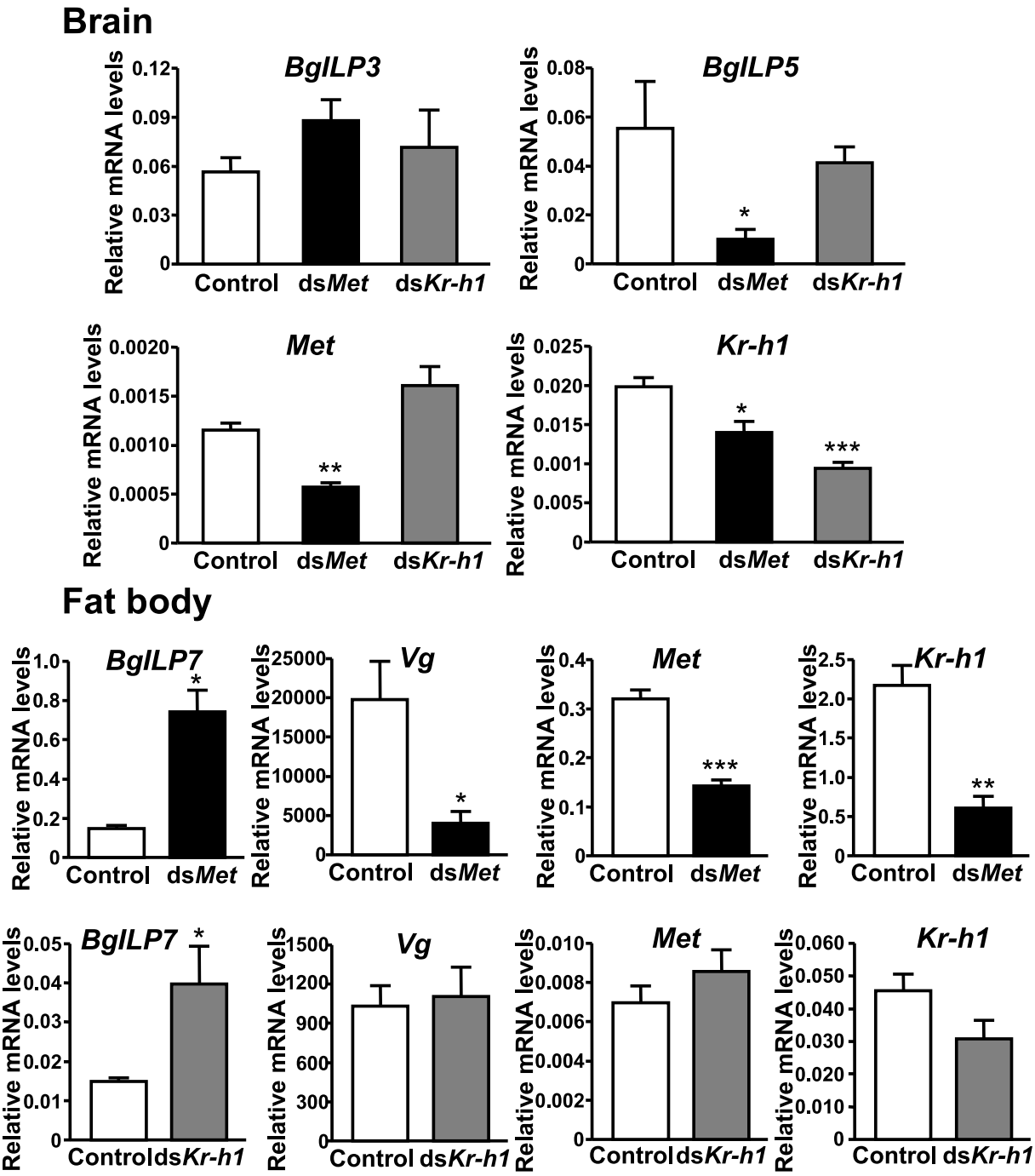

Fig. 5. Effect of *Met* and *Kr-h1* RNAi on brain and fat body. Adult *B. germanica* females were treated with dsRNA targeting *Met* (ds*Met*), *Kr-h1* (ds*Kr-h1*) or a heterologous dsRNA (Control) during the oothecal transport period, and dissections were performed on day 5 of the second gonadotrophic cycle (see Material and Methods). Graphs show *BgILP3*, *BgILP5*, *Met* and *Kr-h1*, mRNA levels in brains (n = 6) and *BgILP7*, *Vg*, *Met* and *Kr-h1* mRNA levels in fat bodies (n = 5–6). *Y*-axes indicate copies per copy of *Actin 5C* in the case of brains and ds*Kr-h1* treated fat bodies and copies per copy of *EIF4a* in the case of ds*Met* treated fat bodies. The results are expressed as the mean ± S.E. Asterisks represent significant differences between Control and treated animals (Student's *t*-test, $*p < 0.05$; $**p < 0.001$; $***p < 0.0001$).

3.5. Effect of the treatment on the IIS

In order to determine the activity of the IIS pathway, we measured *InR* mRNA levels (InR Cluster I according to Smýkal et al., 2020) in the fat bodies of females from the ds*FoxO* starved and the ds*TOR* and ds*JHAMT* treatments. ds*FoxO* treatment in starved females induced a 62% reduction of *InR* mRNA levels, which indicated that the increase in IIS determined by FoxO depletion caused a decrease in *InR* expression (Fig. 6). Both ds*TOR* and ds*JHAMT* treatments also produced a decrease of *InR* mRNA levels, whereas JH treatment in JHAMT-depleted females tended to restore them. Altogether, these results suggested that the changes in ILP levels resulting from JH reduction induced an increase in fat body IIS, while the application of JH and its concomitant change in ILP levels reduced fat body IIS.

4. Discussion

B. germanica ILPs are differentially regulated by nutrition. Starvation

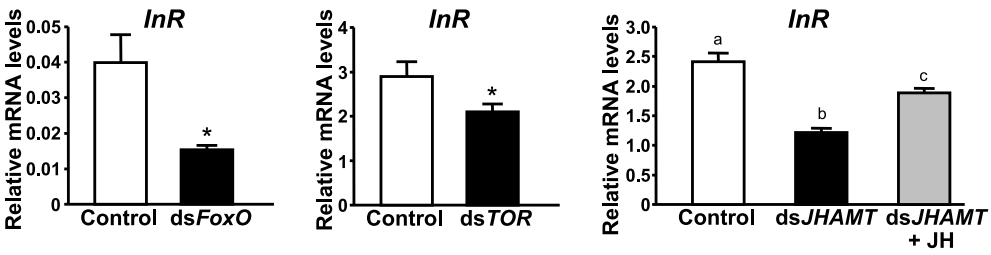

Fig. 6. Effect of *FoxO*, *TOR* and *JHAMT* RNAi on fat body InR expression. Adult *B. germanica* females were treated with dsRNA targeting *FoxO* (ds*FoxO*), *TOR* (ds*TOR*), *JHAMT* (ds*JHAMT*) or a heterologous dsRNA (Control) during the oothecal transport period, and dissections were performed on day 5 of the second gonadotrophic cycle (see Material and Methods). In the case of the ds*FoxO* experiment, the animals were starved from the beginning of the second gonadotrophic cycle. In the case of the ds*JHAMT* experiment, animals were treated 24 h before the dissections with 20 μg JH (ds*JHAMT* + JH), or with acetone (Control and ds*JHAMT*). *Y*-axes indicate copies per copy of *Actin 5C* in the case of the ds*FoxO* experiment and copies per copy of *EIF4a* in the case of the ds*TOR* and ds*JHAMT* experiments. The results are expressed as the mean ± S.E (n = 4–6). Asterisk represents significant differences between Control and treated animals (Student's *t*-test, $*p < 0.05$) and the different letters (a–c) indicate groups with significant differences according to the ANOVA test (Tukey, $p < 0.05$).

elicits an 85–90% reduction in *BgILP3* and *BgILP5* and a ca. 50% reduction in *BgILP6* expression in the brain, a more than 95% reduction in fat body *BgILP7* expression, and an increase in ovarian *BgILP2* mRNA levels (Castro-Arnau et al., 2019). In contrast, no changes are observed in the expression of the other *BgILPs*. These expression changes resulting from starvation are not due to reduced levels of JH, since a treatment with sufficient JH to increase the expression of *Vg* in the fat body is unable to restore the mRNA levels of these *BgILPs* in fed females.

One possibility is that, in adult *B. germanica* females, starvation depletes the IIS pathway by reducing the expression and/or release of some of the *BgILPs*. In turn, this reduction in IIS would produce FoxO dephosphorylation and activation. FoxO would then inhibit the expression of a number of other *BgILPs*. If this were the case, starvation plus FoxO-depletion would produce the increased expression of at least some of the *BgILPs*. The results showed that adding a ds*FoxO* treatment to the starvation did not change *BgILP3* and *BgILP6* expression but, unexpectedly, did reduce that of *BgILP5*. This result suggests that FoxO is necessary for maintaining the low *BgILP5* mRNA levels observed during starvation and preventing these levels from dropping further, in order to preserve necessary, although low, expression levels. In the ds*FoxO* treatment of fed females, *BgILP5* was also the gene that presented a greater than 50% reduction, on average, although the difference with the controls was not statistically significant. In adult *Drosophila melanogaster* females, *dilp3* (but not *dilp2* or *dilp5*) mRNA levels were shown to be reduced in *FoxO* null mutants (Broughton et al., 2008). Also in adult *D. melanogaster* females, but using a different *FoxO* mutant, reduced *dilp2*, *dilp3* and *dilp5* expression was reported (Slack et al., 2011). In addition, *FoxO*-specific activation in the adult pericerebral fat body resulted in increased *dilp2* expression (Hwangbo et al., 2004). In the case of larvae, however, when *FoxO* was specifically depleted in brain Insulin Producing Cells (IPCs), *dilp5* expression was activated (Okamoto and Nishimura, 2015). In the larvae of the beetle *T. castaneum*, ds*FoxO* treatment reduced *ilp-2*, *-3,* and *-4*, although the authors attribute this to the fact that dsFoxO also results in reduced food intake levels (Lin et al., 2018). This cannot be the case in our study since the results were obtained using starved individuals. In the kissing bug, *Rhodnius prolixus*, ds*FoxO* reduces *insulin growth factor* (*IGF*) adult females expression but, unlike *BgILP5*, the expression of *IGF* is greater in unfed than fed females (Leyria et al., 2020, 2021). The differences between the results observed in *B. germanica* compared to those obtained in other species of insects may be due to differences in the experimental protocol, including the fact of using juvenile vs. adult specimens, but also to differences in the specific regulation of the different genes.

The reduced expression of *BgILPs* observed in starved females is also not due to the presumed reduction in TOR pathway signalling that occurs during starvation (Hansen et al., 2004; Maestro et al., 2009; Oldham et al., 2000), since TOR depletion does not restore *BgILP* expression levels. In *D. melanogaster*, several TOR-dependent factors have been reported to send signals from the fat body to the brain IPCs, regulating mainly DILP secretion, but also transcription in some cases (Agrawal et al., 2016; Delanoue et al., 2016; Géminard et al., 2009; Ingaramo et al., 2020; Koyama and Mirth, 2016; Sano et al., 2015).

In *B. germanica*, *TOR* interference results in reduced growth of the developing oocytes (Maestro et al., 2009). In *TOR*-depleted females, ovarian *BgILP2* mRNA levels are similar to those reported during the reproductive cycle in control females with oocytes of the same size (Castro-Arnau et al., 2019). This could correspond to the *BgILP2* expression at that stage of maturation, rather than to TOR acting on its expression.

JH is the gonadotrophic hormone in cockroaches, as well as many other insects (Belles, 2005), and JH synthesis and JH levels in circulation increase throughout the reproductive cycle of *B. germanica* females (Cruz et al., 2003; Maestro et al., 1994). To study the effect of JH on the expression of *BgILPs*, we depleted the expression of *JHAMT*, a gene coding for a key enzyme in JH biosynthesis (Dominguez and Maestro, 2018). JHAMT depletion reduced JH and Vg synthesis as well as follicle growth to extremely low levels (Dominguez and Maestro, 2018; this work). Moreover, reducing JH levels produced a strong decrease in brain *BgILP5* mRNA levels. This reduction tended to be corrected with a treatment with JH 24 h before. In addition, JH depletion produced an increase in brain *BgILP3* mRNA levels, which was again corrected with a JH treatment. BgILP3 and BgILP5 show a kind of compensatory regulation, in the sense that the reduced expression of one of them produces an expression increase of the other (Castro-Arnau et al., 2019), possibly as a way to maintain certain levels of activity of such an important pathway. Thus, depleting JH levels could produce, in the first instance, reduced *BgILP5* expression or increased *BgILP3* expression, and the compensatory regulation would produce the effect on the expression of the other *BgILP*. In the fat body, the reduction of JH produced a huge increase in *BgILP7* expression, the levels of which were reduced after treatment with JH; in the ovary, there was a similar effect on *BgILP2* mRNA levels. In *D. melanogaster*, overexpression of fat body *dilp6* results in a reduction in the expression of brain *dilp2* and *dilp5* (Bai et al., 2012). In *B. germancia*, it is not the increase in BgILP7 what causes the reduction of *BgILP5* expression since *BgILP7* RNAi does not modify the levels of *BgILP5* mRNA (Castro-Arnau et al., 2019).

Thus, the results showed that, in the brain, JH activates the expression of *BgILP5* or inhibits the expression of *BgILP3,* or both, whereas in the fat body, JH inhibits the expression of *BgILP7*. In terms of the ovary, it is possible that the observed levels, as in the case described above, correspond to the physiological and developmental changes that occur as a consequence of the treatments, rather than to the direct effect of JH on the expression of *BgILP2*.

The increase in the mRNA levels of *BgILP3* and *BgILP7* and the slight decrease in those of *BgILP5* observed in the treatment with ds*TOR* could be due to the fact that TOR depletion also reduces the levels of JH (Maestro et al., 2009).

In terms of *BgILP* expression, *Met* depletion produced a phenotype similar to that of *JHAMT* depletion. As expected, this indicates that the effect of JH on *BgILPs* operates through the JH receptor. In the case of *Kr-*

h1, the depletion elicited no effect on the expression of brain *BgILPs*, although, as observed in the treatments with ds*JHAMT* and ds*Met*, ds*Kr-h1* treatment increased fat body *BgILP7* mRNA levels. This indicates that Kr-h1 is involved in regulating the expression of fat body *BgILP7*, but not of that of brain *BgILP3* and *BgILP5*. On the other hand, although the reduction of *Kr-h1* mRNA levels in the fat body is not statistically significant (although sufficient to produce an effect on *BgILP7* expression), we did not observe an effect on *Vg* expression, which could suggest that in *B. germanica*, JH action on *Vg* expression requires Met but not Kr-h1.

In *T. castaneum* adult females, dsRNA-targeting *JHAMT* reduces brain and fat body levels of *ilp2* and *ilp3* mRNAs, whereas JH treatment tends to restore normal levels (Sheng et al., 2011). Also in *T. castaneum*, *ilp2* expression in starved adult males is reduced in *Met* RNAi beetles (Xu et al., 2013). Similarly, in the locust *Schistocerca gregaria*, ds*Met* reduces fat body insulin-related peptide (*SgIRP*) expression (Gijbels et al., 2019). In *D. melanogaster*, a hypomorphic mutant of *Kr-h1* reduces brain *dilp2* and *dilp5* expression (Kang et al., 2017), and in the mosquito *Aedes aegypti*, JH treatment increases *ilp 2*, *6* and *7* and represses *ilp 1*, *3*, *4*, *5* and *8* expression, whereas ds*Met* and ds*Kr-h1* treatment elicited the opposite effect (Ling and Raikhel, 2021). In addition, these authors demonstrated that Kr-h1 binds to the promoter of all the *ilps*, regardless of their activatory or inhibitory effect, while Met only binds to the promoter of *ilp 6* (Ling and Raikhel, 2021).

To ascertain IIS activity, we measured fat body *InR* mRNA levels in different treatments. In *D. melanogaster*, reduction of IIS produces the activation of FoxO, which results in an activation of *InR* expression as a feedback mechanism (Puig et al., 2003; Puig and Tjian, 2005). Also in *B. germanica*, we already demonstrated that starvation increases fat body *InR* mRNA levels whereas ds*FoxO* treatment abolishes this increase (Abrisqueta et al., 2014), which enables the use of *InR* mRNA levels as a marker of IIS activity also in this species. The present results again showed that FoxO depletion and the consequent IIS increase reduced fat body *InR* mRNA levels. In addition, treatments that produced a reduction of JH (ds*TOR* and ds*JHAMT*) showed reduced fat body *InR* mRNA levels whereas JH treatment increased them, which indicated IIS activation and inhibition, respectively. These results suggested that ILPs levels induced by those treatments will be responsible of the changes in IIS, at least in fat bodies.

As we discussed above, RNAi for *JHAMT* in *T. castaneum* reduces *ilp* expression and it is ilp reduction that triggers the decrease in *Vg* transcription (Sheng et al., 2011). This does not appear to be exactly the case in *B. germanica* where, although InR activity is necessary for full activation of *Vg* expression (Abrisqueta et al., 2014), JH reduction increases fat body IIS (this work), while *Vg* expression is low (Dominguez and Maestro, 2018). We then conclude that in *B. germanica*, both JH and IIS are necessary for activating *Vg* expression.

In summary, the reduction of *BgILP3*, *5* and *6* expression in the brain and *BgILP7* in the fat body observed in starvation is not due to low levels of JH or TOR pathway activity, or to the action of FoxO, but to unknown regulatory processes whose activity outweighs the effect of the factors indicated above. *BgILP3* and *BgILP5* are the brain *ILPs* that are subject to more dynamic regulatory activity. In addition, they show more reduced expression in starvation and are those for which compensatory regulation has been demonstrated (Castro-Arnau et al., 2019). On the other hand, it is remarkable that the action of JH on the expression of *BgILP7* in the fat body requires the activity of Kr-h1, while this transcription factor is not necessary in the case of brain *BgILPs*. As for *BgILP2* expression regulation in the ovary, the observed results seem more related to the effect of the treatment on the maturation of the ovaries than to the regulation of *BgILP2* specifically.

The results presented here point to a number of signalling pathways, in particular the FoxO and JH pathways, that contribute to regulating the expression of *B. germanica ILPs*. As discussed above, the results obtained in other species show similar regulation in some cases, although in others the regulation is very different. When analysing the sequences of the different insect ILPs, it has not been possible to identify orthologies between peptides, even when they belong to closely related groups (Antonova et al., 2012; Veenstra, 2020). This suggests that the gene duplications that gave rise to the different ILPs occurred relatively late on in evolution and, in most cases, independently in different lineages. This may have determined the specific regulation of *ILPs*, even in closely related insect groups, making it difficult to find general trends.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2021.103706.

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