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Title: Insulin receptor-mediated nutritional signalling regulates juvenile hormone biosynthesis and vitellogenin production in the German cockroach

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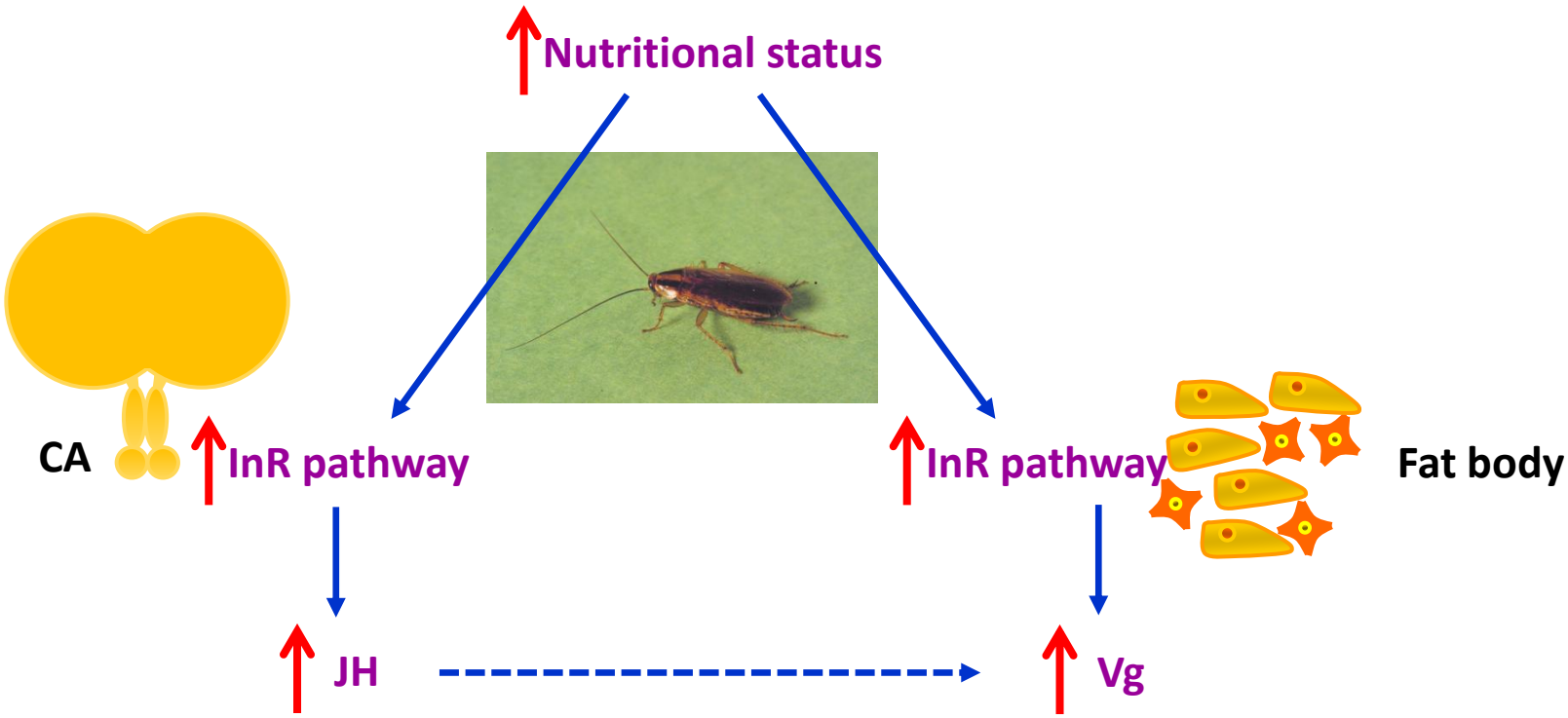
Abstract: Female reproductive processes, which comprise, amongst others, the synthesis of yolk proteins and the endocrine mechanisms which regulate this synthesis, need a considerable amount of energy and resources. The role of communicating that the required nutritional status has been attained is carried out by nutritional signalling pathways and, in particular, by the insulin receptor (InR) pathway. In the present study, using the German cockroach, Blattella germanica, as a model, we analysed the role of InR in different processes, but mainly those related to juvenile hormone (JH) synthesis and vitellogenin production. We first cloned the InR cDNA from B. germanica (BgInR) and then determined that its expression levels were constant in corpora allata and fat body during the first female gonadotrophic cycle. Results showed that the observed increase in BgInR mRNA in fat body from starved compared to fed females was abolished in those females treated with systemic RNAi in vivo against the transcription factor BgFoxO. RNAi-mediated BgInR knockdown during the final two nymphal stages produced significant delays in the moults, together with smaller adult females which could not spread the fore- and hindwings properly. In addition, BgInR knockdown led to a severe inhibition of juvenile hormone synthesis in adult female corpora allata, with a concomitant reduction of mRNA levels corresponding to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1, HMG-CoA synthase-2, HMG-CoA reductase and methyl farnesoate epoxidase. BgInR RNAi treatment also reduced fat body vitellogenin mRNA and oocyte growth. Our results show that BgInR knockdown produces similar phenotypes to those obtained in starved females in terms of corpora allata activity and vitellogenin production, and indicate that the InR pathway mediates the activation of JH biosynthesis and vitellogenesis elicited by nutrition signalling.

*Graphical Abstract (for review)



Highlights

We analysed insulin receptor (InR) function with regard to moult and reproduction in the cockroach *Blattella germanica*.

InR knockdown reduces growth, delays moults and impairs correct wing spread.

In adult females, InR knockdown reduces juvenile hormone (JH) biosynthesis and vitellogenin production.

InR-mediated nutritional signalling is necessary for correct growth and moulting and activates JH and vitellogenin production.

Insulin receptor-mediated nutritional signalling regulates juvenile hormone biosynthesis and vitellogenin production in the German cockroach

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Abstract

Female reproductive processes, which comprise, amongst others, the synthesis of yolk proteins and the endocrine mechanisms which regulate this synthesis, need a considerable amount of energy and resources. The role of communicating that the required nutritional status has been attained is carried out by nutritional signalling pathways and, in particular, by the insulin receptor (InR) pathway. In the present study, using the German cockroach, *Blattella germanica*, as a model, we analysed the role of InR in different processes, but mainly those related to juvenile hormone (JH) synthesis and vitellogenin production. We first cloned the InR cDNA from *B. germanica* (BgInR) and then determined that its expression levels were constant in corpora allata and fat body during the first female gonadotrophic cycle. Results showed that the observed increase in BgInR mRNA in fat body from starved compared to fed females was abolished in those females treated with systemic RNAi *in vivo* against the transcription factor BgFoxO. RNAi-mediated BgInR knockdown during the final two nymphal stages produced significant delays in the moults, together with smaller adult females which could not spread the fore- and hindwings properly. In addition, BgInR knockdown led to a severe inhibition of juvenile hormone synthesis in adult female corpora allata, with a concomitant reduction of mRNA levels corresponding to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1, HMG-CoA synthase-2, HMG-CoA reductase and methyl farnesoate epoxidase. BgInR RNAi treatment also reduced fat body vitellogenin mRNA and oocyte growth. Our results show that BgInR knockdown produces similar phenotypes to those obtained in starved females in terms of corpora allata activity and vitellogenin synthesis, and indicate that the InR pathway mediates the activation of JH biosynthesis and vitellogenin production elicited by nutrition signalling.

Keywords: Insulin receptor, juvenile hormone, vitellogenesis, *Blattella germanica*, nutritional signalling

1. Introduction

Nutrition and reproduction are essential processes for all organisms and have clear interconnections. To be able to reproduce, organisms need to achieve a nutritional status that ensures the viability of the progenitors and that of their progeny. How do the cells and tissues of an organism detect its nutritional status and regulate the processes that will lead to reproduction? The answer is far from simple because many factors (metabolic, endocrine, genetic or environmental) are known to be involved.

The responsibility of communicating the nutritional status of the individual lies mainly with the TOR (target of rapamycin) and insulin receptor (InR) pathways. Both pathways, interconnected at certain points by regulatory factors, are capable of determining the organism's nutritional status and subsequently modulating the activity of a series of effectors that can activate or inhibit different processes depending on factors such as the tissue, the developmental or reproductive timing, etc. Some of the processes regulated by the activity of these pathways are as important as growth, cellular proliferation, metabolism, aging, reproduction and cancer (Baker and Thummel, 2007; Hansen et al., 2004; Maestro et al., 2009; Oldham and Hafen, 2003).

The German cockroach *Blattella germanica* is a basal hemimetabolous insect which presents an anautogenous reproductive strategy. This means that, as is the case for bloodsucking mosquitoes, females do not trigger reproductive processes until after they have fed (Maestro et al., 2009; Osorio et al., 1997). The gonadotrophic hormone in *B. germanica*, as in most insect species, is the juvenile hormone (JH) synthesised in the corpora allata (CA). JH activates vitellogenin production in the fat body and its incorporation into the growing oocytes as a storage protein for embryo development. Previous studies on this cockroach species indicated that nutritional signals that activate JH and vitellogenin production in adult females are mediated, at least partially, by the TOR pathway (Maestro et al., 2009).

The InR pathway is an evolutionary conserved mechanism, present in all metazoan, which detects and responds to changes in nutrient levels (Baker and Thummel, 2007; Oldham and Hafen, 2003; Wu and Brown, 2006). In the fruit fly, *Drosophila melanogaster*, the inhibition of InR signalling phenocopies starvation at a cellular and organismal level (Britton et al., 2002). Insects have a single InR, with the exception of some hymenopteran which have two (de Azevedo and Hartfelder, 2008; Lu

and Pietrantonio, 2011). In contrast to the presence of a single receptor, a variable number of insulin-like peptides (ILPs) can be found in different insect species, for example, eight in *D. melanogaster* (Colombani et al., 2012; Garelli et al., 2012), four in the red flour beetle, *Tribolium castaneum* (Li et al., 2008), and up to thirty-eight in the silkworm, *Bombyx mori* (Aslam et al., 2011). Both the expression and/or release of ILPs are nutritionally regulated in different insect models (Geminard et al., 2009; Ikeya et al., 2002; Masumura et al., 2000; Sheng et al., 2011). In addition, the genetic ablation of brain neurosecretory cells that produce ILPs mimics the phenotype of starved flies (Ikeya et al., 2002; Rulifson et al., 2002). Furthermore, culture media conditioned with cells transfected with *D. melanogaster* ILP genes are able to activate autophosphorylation of the fly InR (Rulifson et al., 2002). It is then clear that the production and release of neurosecretory peptides (ILPs) in response to appropriate nutritional levels is capable of activating the InR and its signalling pathway.

The main transcriptional effector of the InR pathway is the protein FoxO (Barthel et al., 2005). Activation of the InR pathway for example, in the case of high nutritional conditions, phosphorylates FoxO and maintains it inactive within the cytoplasm, whereas starvation promotes the transport of FoxO into the nucleus so that it may perform its transcriptional activities (Baker and Thummel, 2007). We have previously demonstrated that FoxO in *B. germanica* plays an inhibitory role on JH and vitellogenin production in starved females (Suren-Castillo et al., 2012).

In the present work we aim to understand the role of the InR in communicating the organism's nutritional status to different processes, mainly JH and vitellogenin production, and at what levels its regulatory functions are carried out.

2. Material and Methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared on dry dog food (Panlab 125C3) and water, in the dark at 30 ± 1 ºC and 60 - 70% relative humidity. Virgin females were used for the study of gene expression levels during the first gonadotrophic cycle. For the starvation experiments, subjects received only water after the imaginal moult or after the induction of the second gonadotrophic cycle. Dissections of the different tissues were carried out on carbon dioxide-anesthetized specimens. After

dissection, tissues for mRNA levels analysis were immediately frozen in liquid nitrogen and stored at -80 ˚C. Fat body tissue that had adhered to the abdominal sternites was dissected out, except in the case of *in vitro* incubations, where fat body together with the abdominal sternites and epidermis was used.

2.2. Cloning of BgInR

Degenerate primers based on conserved regions of insect and chordate InR sequences were used to obtain a *B. germanica* homologue cDNA fragment by RT-PCR. The first PCR amplification was carried out using a cDNA template generated by reverse transcription of RNA extracted from UM-BGE-1 cells (derived from early embryos of *B. germanica*). The primer sequences are presented in Supplementary Data, Table 1. We amplified a 399 bp fragment, which was subcloned into the pSTBlue-1 vector (Novagen) and then sequenced. This was followed by 3'-RACE and several 5'-RACEs (5'- and 3'-RACE System Version 2.0; Invitrogen) using different specific primers to complete the sequence.

2.3. Phylogenetic analysis

We used sequences from the following insects: *Acyrthosiphon pisum* (GenBank™ Accession Number: XP_001952079), *Aedes aegypti* (AAB17094), *Anopheles gambiae* (XP_320130), *Bombyx mori* (NP_001037011), *Drosophila melanogaster* (AAC47458), *Nasonia vitripennis* (XP_001606180), *Pediculus humanus corporis* (XP_002430961) and *Tribolium castaneum* (EFA11583); the tick *Ixodes scapularis* (XP_002416224); the nematode *Caenorhabditis elegans* (Daf-2: AAC47715); the amphioxus *Branchiostoma lanceolatum* (AAB50848); and the vertebrates *Homo sapiens* InR (AAA59452), *H. sapiens* IGF1R (AAI13611), *Mus musculus* InR (AAA39318) and *M. musculus* IGF1R (NP_034643). The tree was rooted in the divergence between invertebrates and chordates. Protein sequences were aligned using ClustalX (Thompson et al., 1997). Poorly aligned positions and divergent regions were eliminated using Gblocks 0.91b (Castresana, 2000). The resulting alignment was analyzed with the program PHYML 3.0 (Guindon and Gascuel, 2003), based on the maximum-likelihood principle. Four substitution rate categories optimizing the gamma shape parameter were used. The data sets were bootstrapped for 100 replicates.

2.4. RNA extraction, cDNA synthesis and real-time PCR analyses

The CA and fat body expression levels of the different genes studied were analyzed using real-time PCR. cDNA was synthesized from total RNA as described previously (Maestro and Belles, 2006). 0.5 µg of total RNA was used in the case of fat bodies, whereas in the case of CA, the whole RNA from one pair of glands was used. The absence of genomic contamination was confirmed using a control without reverse transcription. cDNA levels were quantified using iQ SYBR Green supermix (Bio-Rad) in an iQ cycler and iQ single colour detection system (Bio-Rad) as described previously (Maestro et al., 2010). Primer sequences to amplify BgInR are reported in Supplementary Data, Table 1. Primers used to amplify HMG-CoA synthase-1 and -2, HMGCoA reductase, methyl farnesoate epoxidase (CYP15A1), vitellogenin (BgVg) and BgActin 5C (used as a reference) have been already reported (Maestro et al., 2010; Suren-Castillo et al., 2012). The total reaction volume was 20 µl. All reactions were run in duplicate or triplicate. The schedule used to amplify the reaction was the following: (i) 95 ºC for 3 min; (ii) 95 ºC for 10 s; (iii) 60 ºC for 1 min; and (iv) repeat steps (i) and (ii) for 50 cycles. Real-time data was collected through the iQ5 optical system software v. 2.0 (BioRad).

2.5. RNA interference

Systemic RNAi *in vivo* in females of *B. germanica* was performed as described previously (Maestro et al., 2009). Two different fragments, a 326-bp dsRNA fragment (dsInR) encompassing part of the protein tyrosine kinase domain of BgInR, and a 349-bp fragment (dsInR-II) encompassing most of the fibronectin type-III domain (spanning positions 3403 to 3728 and 2714 to 3063, respectively, of the BgInR cDNA), were used to generate two different dsRNA (Fig. 1A). A heterologous 307-bp fragment from the polyhedrin of *Autographa californica* nucleopolyhedrovirus (dsControl) was used as a control. A dose of 2 µg of dsRNA diluted in sterile saline was injected into the abdomen of freshly emerged penultimate (fifth) nymph instar females, followed by a second 2 µg dose injected when they moulted into the last (sixth) instar. Dissections were carried out 5 days after the adult moult. In another set of RNAi experiments, adult females in the first day of ootheca transport were treated with a single 2 µg dsRNA dose. Twelve days later, the ootheca was removed, which induced the onset of the second gonadotrophic cycle, and dissections were carried out 5 days later. The mRNA levels of BgInR, HMG-

CoA synthase-1 and -2, HMG-CoA reductase, methyl farnesoate epoxidase, BgVg and BgActin 5C (used as a reference) were determined by RT-qPCR.

RNAi treatment for silencing BgFoxO was performed as described (Suren-Castillo et al., 2012, 2014).

2.6. Quantification of juvenile hormone biosynthesis

JH III biosynthesis by CA incubated *in vitro* was quantified according to the method reported previously (Maestro et al., 2009). Essentially, individual pairs of CA were incubated in 100 µl of 199 medium (Sigma) containing L-methionine (0.1 nM), Hank's salts, Hepes buffer (20 mM) and Ficoll (20 mg/ml), to which L-[3 H-*methyl*] methionine (Perkin Elmer) was added to achieve a final specific activity of 7.4 GBq/mmol. CA were incubated for 3 h, after which JH III was quantified in the medium plus homogenized glands.

2.7. Incubation of fat body in vitro

Fat body tissue that had adhered to abdominal tergites and epidermises was dissected from freshly emerged adult females. The fat body tissue was then preincubated for 30 min. in 1 ml of Grace's medium, with L-glutamine and without insect haemolymph (Sigma) at 30 °C in the dark, as described previously (Maestro et al., 2009). After preincubation, tissues were incubated for 4 h in media supplemented with 50 µM LY294002 (Calbiochem) or the corresponding volume of DMSO (LY294002 solvent) and posteriorly transferred to media containing, in addition to the previous treatment, 800 nM JH III or the corresponding volume of acetone (JH solvent) and incubated for a further 6 h. After final incubation, tissues were frozen in liquid nitrogen and stored until RNA extraction.

2.8. Measurement of food intake

Food intake was measured as reported previously (Pascual et al., 2008) with some minor modifications. Briefly, individual specimens (females on their first day into the penultimate nymphal instar, treated with either dsControl or dsInR) were provided with a portion of food (dry dog food) of known mass, and, just when they moulted into adult, the remaining food was dried in an oven and its mass recorded. The water lost due to evaporation from a similar portion of food placed in a control box, containing only the water vial, was used as a correction factor.

3. Results

3.1. Cloning of BgInR, sequence comparison and phylogenetic analysis

Using degenerate primers and cDNA from *B. germanica* UM-BGE-1 cells as a template, a 399 bp fragment sequence of a presumed InR homologue from *B. germanica* (BgInR) was obtained. To complete the cDNA sequence, we followed 3'-RACE and 5'-RACE methodologies and obtained a sequence of 4562 bp (GenBank accession number: HG518668), which encoded a protein of 1403 amino acids. The putative start codon was preceded by an in-frame stop codon and the final stop codon was followed by a polyA sequence, suggesting that it was the full-length open reading frame. BLAST and Pfam database searches indicated that the protein was the *B. germanica* homologue of InR. BgInR contains a protein tyrosine kinase catalytic domain, typical of InR proteins. The protein also contained a fibronectin type III domain (present in several extracellular animal proteins), two ligand binding domains and a furin-like cysteine rich region, which is characteristic of the extracellular domain of the InR protein (Fig. 1A).

Using the BgInR sequence, and other representative InR sequences available in databases, a maximum-likelihood analysis was performed. The topology of the tree (Fig. 1B) is similar to the current phylogeny of the included species, and indicates that the *B. germanica* sequence corresponds to an InR protein. The branch length corresponding to the *C. elegans* InR homologue (Daf-2) suggests a rapid rate of divergence with respect to other metazoa. The occurrence of vertebrate insulin and IGF1 receptors clustering together indicates that these two molecules diverged after the separation of vertebrates and invertebrates. In addition, the remarkably short length of vertebrate branches indicates the great conservation of these sequences compared to those of invertebrates.

3.2. BgInR expression patterns

mRNA levels of BgInR were analysed in CA and fat body of adult females throughout the first gonadotrophic cycle. BgInR mRNA levels were practically constant in both tissues throughout the first gonadotrophic cycle (Fig. 2A & B). We also quantified BgInR mRNA levels in 5-day old fed and starved adult females. Results

showed no differences in BgInR mRNA levels in CA, whereas higher BgInR mRNA levels were found in fat bodies from starved compared to fed females (Fig. 2C).

3.3. Effects of BgInR RNAi on development

In order to assess BgInR function, its expression was reduced using systemic RNAi. A dose of 2 µg of a 326 bp dsRNA fragment, encompassing part of the tyrosine kinase catalytic domain (dsInR, Fig. 1A), was injected into the abdomen of freshly emerged fifth (penultimate) instar female nymphs, and this treatment was then repeated immediately after the next moult (dsInR group). Specimens treated with a non-homologous dsRNA were used as the control (dsControl group). The effect dsRNA had upon reducing BgInR mRNA was checked by quantifying BgInR mRNA in CA and fat body from 5-day old adult females. The levels in the dsInR group compared to the dsControl group were 74 and 85% lower in CA and fat body, respectively (Fig. 3A).

dsInR nymphs moulted later into the last instar than the dsControl (penultimate instar length, dsControl: 6.22 ± 0.11 days; dsInR 7.00 ± 0.15 days (mean ± SEM); $p < 0.001$, Student's *t*-test), and they also moulted into adults later (last instar length, dsControl: 8.47 ± 0.20 days; dsInR: 9.77 ± 0.34 days; $p < 0.001$, Student's *t*-test) (Fig. 3B). Thus, on average, dsInR females accumulated two days of delay in their development in comparison with the control group.

Further to the above, when the last moult occurred and adult females emerged, almost half of the dsInR females (24 of 51) showed wing and tegmina malformations. Apparently, those cockroaches were neither able to spread their tegmina properly nor could they extend their wings at all (Fig. 4).

To quantify size differences between the different treatment groups, pronotum lengths were measured. Results showed that pronotum growth during the penultimate and last instars was significantly smaller in dsInR female nymphs than in the dsControl group (Fig. 5A). To be sure that the size differences were not a consequence of defective nutrition, the food intake, measured as the amount of dry food ingested, was measured. We found that the food intake of BgInR knockdown females did not differ significantly from that of the controls (Fig. 5B).

3.4. Effects of BgInR RNAi on reproductive processes

JH synthesis in CA from dsControl and dsInR 5-day old adult females was measured. Results showed that BgInR knockdown produced a dramatic reduction in JH

biosynthesis (Fig. 6A). Based on these results, we measured the CA mRNA levels of some of the enzymes in the JH biosynthetic pathway, namely 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase-1, HMG-CoA synthase-2, HMG-CoA reductase and methyl farnesoate epoxidase. CA from the BgInR knockdown group showed significant reductions in the mRNA levels of HMG-CoA synthase-1 (58%), HMG-CoA synthase-2 (63%), HMG-CoA reductase (51%) and epoxidase (84%) compared to the dsControl group, similar to those produced in the case of starved females (Fig. 6B).

Since JH stimulates vitellogenin production, fat body BgVg mRNA levels of BgInR knockdown females were analysed and compared with the levels of control and starved females. BgInR knockdown fat bodies showed a significant 80% reduction in BgVg expression, whereas BgVg mRNA levels in fat body from starved females were practically null (Fig. 7A). Consequently, whereas ovaries from the control group grew and developed normally, those from the BgInR knockdown group did not grow at all and the basal oocytes were as small as those of starved or freshly emerged adult females (Fig. 7B).

3.5. Effects of LY294002 on vitellogenin transcription

Our experiments indicated that BgInR knockdown inhibited JH production in the CA and BgVg expression in the fat body. Considering this, we questioned whether BgVg mRNA reduction in dsInR-treated females was a result of the decrease in JH levels or if BgInR knockdown could also directly affect BgVg transcription independent of JH action. To answer this question, and considering that phosphatidylinositol 3-kinase (PI3K) initiates InR signalling, we incubated fat body tissues *in vitro* in the presence of the PI3K specific inhibitor LY294002. Fat bodies from freshly emerged adult females (which do not yet produce Vg because their CA are inactive) were incubated for 4 h in Grace's medium containing 50 µM of LY294002 or in the corresponding solvent alone (DMSO). The tissues were then transferred to a medium containing 50 µM of LY294002, as previously, plus 800 nM of JH III or the corresponding JH solvent alone (acetone) and incubated for 6 h. Results showed that fat bodies from freshly emerged females incubated in media without JH did not express BgVg, as expected. On the other hand, fat bodies incubated with JH produced BgVg

289 mRNA, but these BgVg expression levels were reduced by the presence of LY294002
290 in the JH treatment (Fig. 7C).

291 *3.6. Effects of a second dsRNA InR*

292 A second RNAi (dsInR-II), based on a 349 bp fragment that encompasses the
293 fibronectin type III domain, was used to perform experiments equivalent to those
294 described above and produced similar phenotypes. BgInR mRNA levels were
295 significantly reduced, both in CA and fat body (supplementary data, Fig. 1A). In
296 addition, pronota from dsInR-II females were smaller than those from dsControl
297 females (supplementary data, Fig. 1B); the expression levels of the enzymes of the JH
298 biosynthetic pathway, HMG-CoA synthase-1, synthase-2 and reductase decreased
299 (supplementary data, Fig. 1C - methyl farnesoate epoxidase mRNA levels were not
300 measured in this experiment); BgVg expression in fat body was also reduced
301 (supplementary data, Fig. 1D); and, consequently, basal oocyte lengths were shorter
302 (supplementary data, Fig. 1E).

303 *3.7. BgInR RNAi treatment in adult females*

304 The treatment of nymphs with dsInR inhibited JH synthesis in adult CA and the
305 expression of BgVg in fat body. However, it also delayed development, reduced adult
306 size and caused wing and tegmina malformation. Whilst bearing this in mind, the
307 possibility still remained that reduced InR levels had affected CA and fat body
308 development. To confirm whether the effects of InR knockdown on JH synthesis and
309 BgVg expression are specific and independent of developmental effects, RNAi
310 experiments were conducted in adult cockroaches. Thus, a dose of 2 µg of dsInR (or
311 dsControl) was injected in adult females on the first day of ootheca transport. After
312 removing the ootheca 12 days later, a second gonadotrophic cycle was induced.
313 Dissections were performed at day 5 of this second cycle. Again, BgInR mRNA levels
314 in the dsInR group were clearly lower than in the control group, both in CA (dsControl:
315 0.153 ± 0.022 (n = 11); dsInR: 0.060 ± 0.015 (n = 12); $p = 0.0018$ Student's *t*-test) and
316 fat body (dsControl: 0.180 ± 0.034 (n = 12); dsInR: 0.011 ± 0.002 (n = 12); $p < 0.0001$
317 Student's *t*-test). Analysis of parameters related to reproduction produced similar results
318 to those observed when nymphs were treated and analysed during the first
319 gonadotrophic cycle. Thus, expression levels of JH biosynthetic enzymes in CA (Fig.

8A) and BgVg in fat body (Fig. 8B), together with basal oocyte lengths (Fig. 8C), were clearly lower in dsInR compared to dsControl-treated adult females.

3.8. Effect of BgFoxO on BgInR expression

We used adult females to analyse the effect of the transcription factor FoxO on BgInR expression. We treated females on the first day of ootheca transport with 2 µg of BgFoxO dsRNA and induced a second gonadotrophic cycle by removing oothecae after 12 days. Results showed that BgFoxO RNAi treatment induced a reduction of BgInR mRNA levels in normally fed females and that the increased BgInR mRNA levels observed in starved dsControl animals were significantly reduced in starved dsFoxO subjects to levels comparable with the fed dsControl group (Fig. 9).

4. Discussion

In the present work we report the cloning of the *B. germanica* orthologue of the InR (BgInR) and the study of its function on certain developmental and reproductive processes. BgInR displays the characteristic organisation of InR/IGF-IR proteins, with two ligand binding domains separated by a furin-like cysteine rich region, followed by a fibronectin type-III domain and a protein tyrosine kinase domain (Lu and Pietrantonio, 2011; Ward and Lawrence, 2009).

BgInR mRNA levels in the CA and the fat body of *B. germanica* adult females remain constant through the first gonadotrophic cycle. To date, few results showing InR mRNA profiles in adult tissues have been reported. In *A. aegypti*, ovary InR mRNA levels gradually increase during the first days after adult eclosion, remain constant during the previtellogenic period and peak immediately after a blood meal and after oviposition (Riehle and Brown, 2002). Although BgInR is expressed in *B. germanica* ovaries (results not shown), we have not fully studied the BgInR mRNA profile in ovaries because our interest is focused on vitellogenesis and their main actors, CA and fat body.

We also measured the effect of starvation on BgInR expression by comparing BgInR mRNA levels in CA and fat body from 5-day old fed and starved adult females. Results showed no significant differences in CA, but significantly higher levels of BgInR mRNA were observed in fat body from starved compared to fed females. Similar

differences between starved and fed conditions were reported in *B. mori* larvae fat body (Liu et al., 2010), *Manduca sexta* prothoracic glands (Walsh and Smith, 2011) or *D. melanogaster*, in both S2 cells and whole flies (Puig et al., 2003; Puig and Tjian, 2005). In addition, higher levels of InR mRNA in CA from starved compared to fed *A. aegypti* adult females, which leads to higher insulin sensitivity in a JH synthesis *in vitro* assay, have been recently reported (Perez-Hedo et al., 2014). Conversely, InR mRNA levels measured in the whole body of the adult red flour beetle, *T. castaneum*, show a clear reduction in starvation (Parthasarathy and Palli, 2011). Furthermore, it was demonstrated that in a situation of limited nutrients, the transcription factor FoxO binds to the *D. melanogaster InR* promoter and activates its expression, with the concomitant increase of InR protein (Puig et al., 2003; Puig and Tjian, 2005). Authors postulate that this process leads to an increase of insulin sensitivity which would permit a very fast activation of the InR pathway as soon as a high nutrient situation is once again attained and insulin-like peptides synthetised (Puig et al., 2003; Puig and Tjian, 2005). We have demonstrated that in *B. germanica* the starvation-induced increase in fat body BgInR mRNA was not produced when BgFoxO expression was silenced using RNAi methodologies, also indicating in our model that FoxO is required to activate BgInR expression during starvation. Also in *A. aegypti*, the observed InR mRNA increase in thorax from starved compared to fed adult females is abolished in FoxO-depleted animals (Perez-Hedo et al., 2014).

Treating penultimate and last instar nymphs with dsInR causes an accumulated delay of approximately two days in the development time until the adult moult. In addition, the size of adult specimens, measured as pronotum length, was smaller in dsInR-treated females compared to the controls. This size reduction cannot be explained by a reduction in food consumption. In the cricket, *Gryllus bimaculatus*, nymphal dsInR treatment reduces the weight of adults, although no differences were found in the length of the development period (Dabour et al., 2011). Nevertheless, RNAi treatment against the cricket orthologue of vertebrate insulin receptor substrate, Chico, does induce a delay in development (Dabour et al., 2011). *D. melanogaster* InR hypomorphic mutants have extended development time and growth deficiencies (Brogiolo et al., 2001; Chen et al., 1996; Tatar et al., 2001). Furthermore, it has been demonstrated that when InR mRNA is reduced after attaining the critical size, the final body size of the fly is affected without affecting development time, whereas reducing InR mRNA before the

384 critical size is reached does not have any extra effect on size reduction but increases
385 development time (Shingleton et al., 2005). In the case of cockroaches, the existence of
386 a critical size has not yet been demonstrated.

387 In addition to longer nymphal stages and smaller adults, in approximately half of
388 the cockroaches, dsInR treatment produces a phenotype characterised by an impaired
389 extension of the tegmina, the sclerotized forewings, and no extension at all of the
390 membranous hindwings. These anatomical deficiencies coincide with the observed
391 phenotype in the case of *B. germanica* nymphs treated with dsRNA against ecdysone
392 receptor isoform-A (Cruz et al., 2006), and point to a reduction in the activity of the
393 ecdysone signalling pathway. In fact, an increase in the duration of nymphal stages
394 would also be compatible with a reduction in the activity of the ecdysone pathway. One
395 possible explanation for a reduction in the ecdysone signalling pathway in dsInR
396 nymphs is that InR RNAi treatment would cause reduced growth of the prothoracic
397 gland, the gland responsible for ecdysone synthesis in cockroach nymphs. This growth
398 reduction would result in lower levels of ecdysone in circulation and, consequently, a
399 reduction in the signalling pathway at the level of the target tissues. Again in the fruit
400 fly, it has been demonstrated that a reduction of insulin signalling specifically in the
401 prothoracic gland produces smaller glands which synthesise less ecdysone, and
402 conversely, an increase in the insulin signal in the prothoracic gland produces enlarged
403 glands which synthetise more ecdysone (Caldwell et al., 2005; Colombani et al., 2005;
404 Mirth et al., 2005).

405 dsInR treatment in penultimate and last instar nymphs almost completely
406 inhibits the synthesis of JH-III (the JH of cockroaches and of most insect species).
407 Together with the reduction in JH synthesis, a decrease in CA mRNA levels of enzymes
408 belonging to the JH biosynthetic pathway has been observed, both in the mevalonate
409 synthesis (HMG-CoA synthase-1 and synthase-2, and reductase) and in the JH specific
410 pathway (methyl farnesoate epoxidase). These reductions are similar to those observed
411 in starved adult females. The reduction observed in JH synthesis in dsInR-treated (this
412 paper) and starved females (Maestro et al., 2009) could be explained by the reduced
413 mRNA levels of the before mentioned enzymes. A reduction in JH biosynthesis has also
414 been reported for *D. melanogaster* InR hypomorphic mutants in studies which
415 demonstrated that InR mutation particularly reduces the levels of JH-III bisepoxide, the
416 major JH subtype in the fly (Tatar et al., 2001; Tu et al., 2005). Also in the fruit fly,

417 silencing InR within the CA results in a reduction of CA HMG-CoA synthase
418 (Belgacem and Martin, 2007). In the mosquito *A. aegyti*, there is a 2 to 3 fold increase
419 in JH-III synthesis when CA from sugar fed females are incubated with bovine insulin
420 (Perez-Hedo et al., 2013), which again indicates that activation of the insulin pathway is
421 necessary for JH synthesis. The same study revealed that incubation with the
422 phosphoinositide-3 kinase (PI3K) specific inhibitor LY294002, which inhibits the
423 transduction of the InR pathway, produces a reduction in JH synthesis and in the
424 expression of the enzymes involved in its biosynthesis, including HMG-CoA synthase,
425 reductase and methyl farnesoate epoxidase. Nevertheless, there is a difference between
426 these models and *B. germanica*; whereas JH is the gonadotrophic hormone of
427 cockroaches (as for most insects), the dipterans gonadotrophic hormone is 20-
428 hydroxyecdysone.
429 BgInR RNAi-treated females also showed low levels of vitellogenin (BgVg)
430 mRNA, concomitant with practically no growth of the basal oocytes. Similarly, in the
431 mosquito *A. aegypti*, dsInR inhibits insulin-induced Vg gene transcription (Roy et al.,
432 2007) delays the appearance of Vg in fat body and reduces the number of follicles per
433 ovary (Gulia-Nuss et al., 2011). Also, in the red flour beetle, *T. castaneum*, dsInR
434 reduces Vg mRNA (Parthasarathy and Palli, 2011), and in the desert locust,
435 *Schistocerca gregaria*, dsRNA treatment against the only ILP described in this species
436 reduces Vg transcription and oocyte length (Badisco et al., 2011).
437 As stated earlier, JH is the gonadotrophic hormone of cockroaches and in *B.*
438 *germanica* it is capable of stimulating the production of Vg at mRNA and protein
439 levels, both *in vivo* (Comas et al., 1999) and *in vitro* (Comas et al., 2001). To answer the
440 question of whether *B. germanica* vitellogenesis is dependent on the InR pathway
441 independently of JH action, we incubated fat bodies from recently emerged adult
442 females, which were not yet vitellogenic and whose CA were inactive, with JH III and
443 with LY294002, a PI3K specific inhibitor, in order to eliminate InR signalling. Our
444 results showed that, whereas JH III was able to activate BgVg mRNA synthesis,
445 LY294002 abolished this induction, demonstrating that the InR pathway must be active
446 for JH-induced vitellogenin production. In *T. castaneum*, InR RNAi inhibits JH
447 induction of Vg mRNA, however JH acid methyltransferase RNAi treatment did not
448 suppress the induction of Vg mRNA synthesis after treatment with bovine insulin
449 (Sheng et al., 2011). In the same study, JH treatment induced the expression of at least

one of *T. castaneum*'s insulin-like peptides (ILPs). Altogether this led the authors to conclude that JH regulates Vg gene expression in *T. castaneum* by inducing the expression of their ILPs. Whether or not this model could be applied to *B. germanica* should be tested directly. In the mosquito *A. aegypti*, although vitellogenesis is sensitive to LY294002, neither insulin alone nor 20-hydroxyecdysone alone could activate Vg transcription (Roy et al., 2007).

Individuals treated with a second BgInR dsRNA (dsInR-II), designed against another part of BgInR mRNA, showed similar phenotypes to those presented up until now: adult females were smaller, mRNA levels of JH biosynthetic enzymes in CA and of BgVg in fat body were reduced, as was basal oocyte growth. These results validate those obtained using the first dsRNA and discard the possibility that they were produced by off-target effects.

dsInR treatment in adults also produced a reduction of mRNA enzymes of the JH biosynthetic pathway in CA, together with an inhibition of BgVg expression and oocyte growth. This indicates that the effects observed in nymphal dsInR treatments related to reproduction were not due to developmental defects and that InR is genuinely necessary to activate JH and Vg synthesis in adult *B. germanica* females.

Considering all of our results together, they indicate that, besides an effect on growth and a possible action on ecdysone synthesis or signalling, InR knockdown in *B. germanica* produces a reduction in JH biosynthesis and vitellogenin production, phenocopying, in this sense, the effect of starvation. Therefore, the results suggest that InR is required to activate JH and vitellogenin synthesis in response to a positive nutritional status. A study on *D. melanogaster* has also reported that PI3K activity is nutritionally regulated and that the inhibition of InR/PI3K signalling phenocopies the effects of starvation (Britton et al., 2002). Further to this, the expression of some DILPs (*Drosophila* insulin-like peptides) is regulated by nutrient availability and the genetic ablation of neurosecretory cells that produce DILPs mimics the phenotype of starved flies (Ikeya et al., 2002; Rulifson et al., 2002). The model proposed by some authors involves the fat body, which would sense the availability of nutrients and would activate the secretion of brain DIPLs through a yet unidentified humoral factor (Geminard et al., 2009).

The mechanism by which activation of the InR pathway stimulates JH and vitellogenin production in *B. germanica* females is still unknown. Nevertheless, the role

of the transcription factor FoxO, the main transcriptional effector of the InR pathway, must be considered. In fact, BgFoxO RNAi treatment in starved *B. germanica* females increases JH biosynthesis and vitellogenin production at mRNA and protein levels (Suren-Castillo et al., 2012), which indicates that BgFoxO is inhibiting these processes during starvation. In *T. castaneum* FoxO knockdown also increases vitellogenin expression and FoxO binding to a FoxO-response element in the vitellogenin promoter has been demonstrated (Sheng et al., 2011). However, BgFoxO RNAi treatment of *B. germanica* starved females doesn't fully recover the JH and vitellogenin levels observed in fed control females (Suren-Castillo et al., 2012), which points to the existence of some other regulatory mechanisms involved in these processes. In *B. germanica*, our research group demonstrated that the kinase TOR is also involved in the activation of JH and vitellogenin production in response to a positive nutritional condition (Maestro et al., 2009). Thus, both InR and TOR pathways mediate the nutritional signals that activate JH biosynthesis in the CA and vitellogenin production in the fat body of adult cockroaches.

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Figure legends

Fig. 1. *B. germanica* InR structure and phylogenetic analysis. (A) BgInR domain organization showing the regions used to generate the two dsRNAs: dsInR and dsInR-II. (B) Phylogenetic tree constructed using the maximum-likelihood approach. Branch lengths are proportional to sequence divergence. The bar represents 0.5 substitutions per site. Bootstrap values (100 replicates) are shown in the nodes. The root of the tree is placed at the divergence between invertebrates and chordates.

Fig. 2. Expression patterns of BgInR mRNA in CA and fat body of *B. germanica* adult females. BgInR mRNA levels in CA (A) and fat body (B) during the 8 days of the first gonadotrophic cycle (n = 3). (C) BgInR mRNA levels in CA (n = 10 - 17) and fat body (n = 9 - 17) from 5 day-old fed and starved *B. germanica* females. The y-axis represents copies per copy of BgActin 5C. Results are expressed as the mean ± S.E. Asterisk represents significant differences between fed and starved subjects (Student's *t*-test, $*P < 0.05$).

Fig. 3. Effect of BgInR RNAi on developmental time. dsRNA targeting BgInR (*dsInR*) or a non-homologous dsRNA (*dsControl*) was administered on the first day of the penultimate (fifth) and last (sixth) nymph instars. (A) BgInR mRNA levels in CA and fat body (n = 17). Dissections were performed five days after the adult moult. The y-axis represents copies per copy of BgActin 5C. Results are expressed as the mean ± S.E. Asterisk represents significant differences between dsControl and dsInR (Student's *t*-

676 test, ***$P < 0.0001$). (B) Cumulative percentage of *B. germanica* females receiving the
677 indicated treatment which moulted into the next stage (sixth nymphal instar or adult).
678 The penultimate nymphal instar lasts 6.22 ± 0.11 days (mean ± S.E.) for the dsControl
679 group (n = 41) and 7.00 ± 0.15 days for dsInR (n = 37) ($P < 0.001$ Student's *t*-test). The
680 last nymphal instar lasts 8.47 ± 0.20 days for dsControl (n = 32) and 9.77 ± 0.34 for
681 dsInR group (n = 22) ($P < 0.001$ Student's *t*-test).

682 Fig. 4. Effect of BgInR RNAi on wings and tegmina extension. Experimental procedure
683 was the same as in Fig. 3. (A) Dorsal view of a dsControl female. (B) Dorsal view of a
684 dsInR female with wings and tegmina not properly extended. (C) Detail of wings of
685 dsInR female after tegmina removal. (D) Wings and tegmina of dsControl female. (E)
686 Malformed tegmina and not extended wings of dsInR female. Scale bars: 2 mm.

687 Fig. 5. Effect of BgInR RNAi on growth. Experimental procedure was the same as in
688 Fig. 3. (A) Pronotum growth during the penultimate and last nymphal instars in
689 dsControl (n = 65) and dsInR females (n = 34). (B) Total food intake during the
690 penultimate and last nymphal instars (n= 7 - 9). Results are expressed as the mean ±
691 S.E. Asterisk represents significant differences between dsControl and dsInR subjects
692 (Student's *t*-test, ***$P < 0.0001$).

693 Fig. 6. Effect of BgInR RNAi on JH synthesis and on mRNA levels of JH biosynthesis
694 pathway enzymes. Experimental procedure was the same as in Fig. 3. Dissections were
695 performed five days after the adult moult. (A) Rates of JH synthesis by CA incubated *in*
696 *vitro* (n= 6 - 9). (B) mRNA levels of HMG-CoA synthase-1, -2, HMG-CoA reductase (n
697 = 15) and methyl farnesoate epoxidase (n = 7). The y-axis represents copies per copy of
698 BgActin 5C. In this case, part of the dsControl group only received water after the adult
699 moult (*starved*). Results are expressed as the mean ± S.E. Asterisk represents significant
700 differences between dsControl and dsInR subjects (Student's *t*-test, **$P < 0.001$). The
701 different letters (a-b) represent groups with significant differences according ANOVA
702 test (Tukey, $P < 0.0001$).

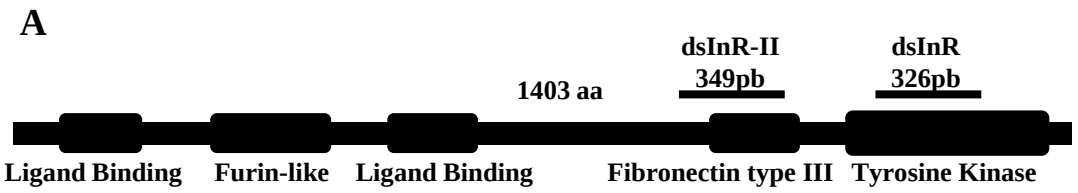
703 Fig. 7. Effect of BgInR RNAi on vitellogenesis. (A) and (B) Experimental procedure
704 was the same as in Fig. 3. Dissections were performed five days after the adult moult.
705 Again, part of the dsControl group only received water after the adult moult (*starved*)
706 (A) BgVg mRNA levels in fat bodies (n = 7 - 17). (B) Basal oocyte lengths (n= 14). (C)
707 Effect of LY294002 on JH induction of BgVg expression. Fat bodies from freshly

emerged adult females were incubated in vitro with different combinations of LY294002 and JH-III (n= 7). *pre* indicates tissues that were only preincubated with control media. In (A) and (C), the y-axis represents copies per copy of BgActin 5C. Results are expressed as the mean ± S.E. The different letters (a-b) represent groups with significant differences according ANOVA test (Tukey, $P < 0.05$).

Fig. 8. Effect of BgInR RNAi on adult females of *B. germanica*. dsRNA targeting BgInR (*dsInR*) or a non-homologous dsRNA (*dsControl*) was administered in the first day of ootheca transport. The ootheca was removed 12 days letter and a second gonadotrophic cycle was triggered. Dissections were performed 5 days later. (A) mRNA levels of HMG-CoA synthase-1, -2, HMG-CoA reductase and methyl farnesoate epoxidase (n = 10). (B) BgVg mRNA levels in fat bodies (n=10). The y-axis represents copies per copy of BgActin 5C. (C) Basal oocyte lengths (n = 15). Results are expressed as the mean ± S.E. Asterisks represent significant differences between dsControl and dsInR subjects (Student's *t*-test, $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$).

Fig. 9. Effect of starvation and BgFoxO RNAi (dsFoxO) on fat body BgInR expression. mRNA levels of BgInR were analyzed in fat bodies from fed and starved dsControl (n= 14 - 16) and dsFoxO (n = 10 - 16) females. Experimental procedure was the same as in Fig. 8, except treatment was performed with BgFoxO RNAi. Starved females received only water after the ootheca was removed and dissections were carried out 5 days later. The y-axis represents copies per copy of BgActin 5C. Results are expressed as the mean ± S.E. The different letters (a-c) represent groups with significant differences according ANOVA test (Tukey, $P < 0.05$).

Figure 1


A
dsInR-II
349pb
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1403 aa
Ligand Binding
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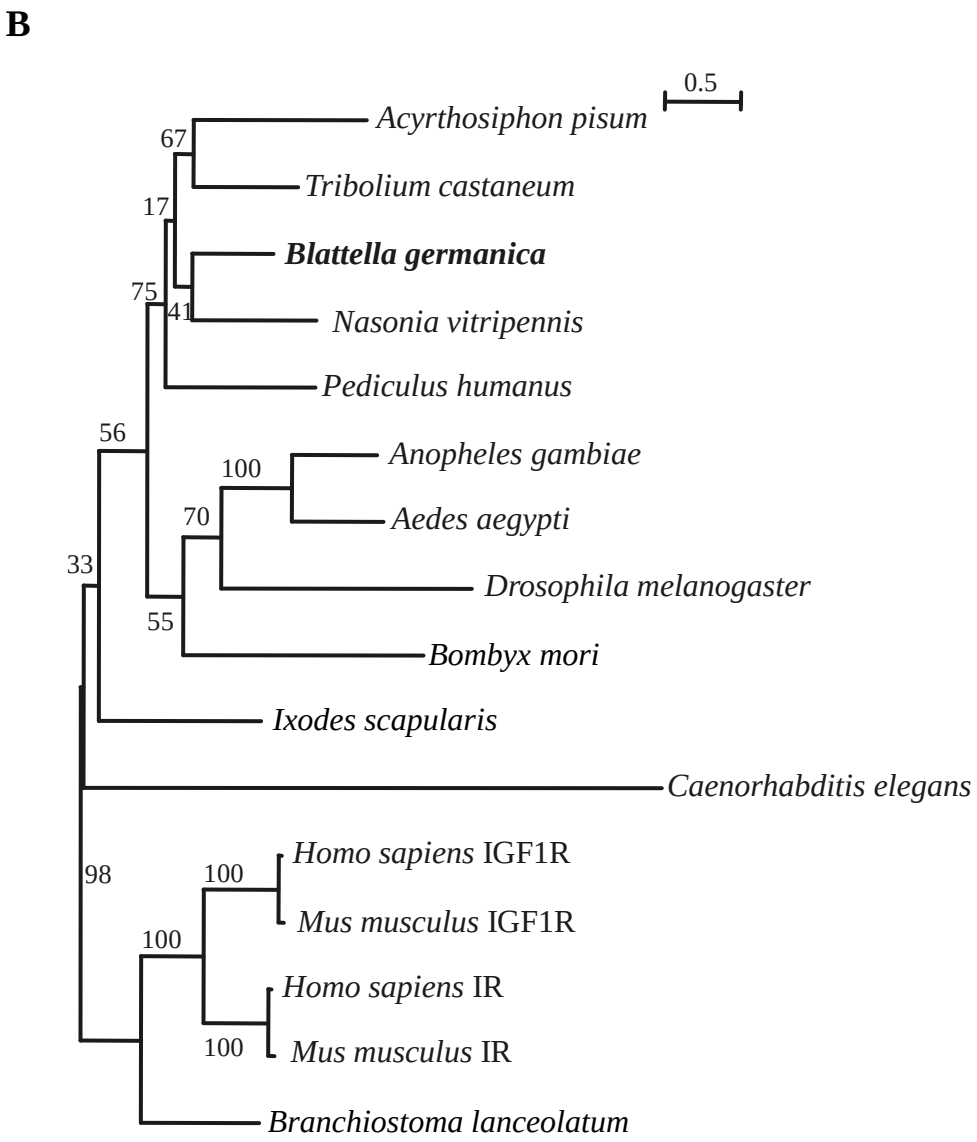
Tyrosine Kinase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0.5
Acyrthosiphon pisum
Tribolium castaneum
Blattella germanica
Nasonia vitripennis
Pediculus humanus
Anopheles gambiae
Aedes aegypti
Drosophila melanogaster
Bombyx mori
Ixodes scapularis
Caenorhabditis elegans
Homo sapiens IGF1R
Mus musculus IGF1R
Homo sapiens IR
Mus musculus IR
Branchiostoma lanceolatum
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Figure 2

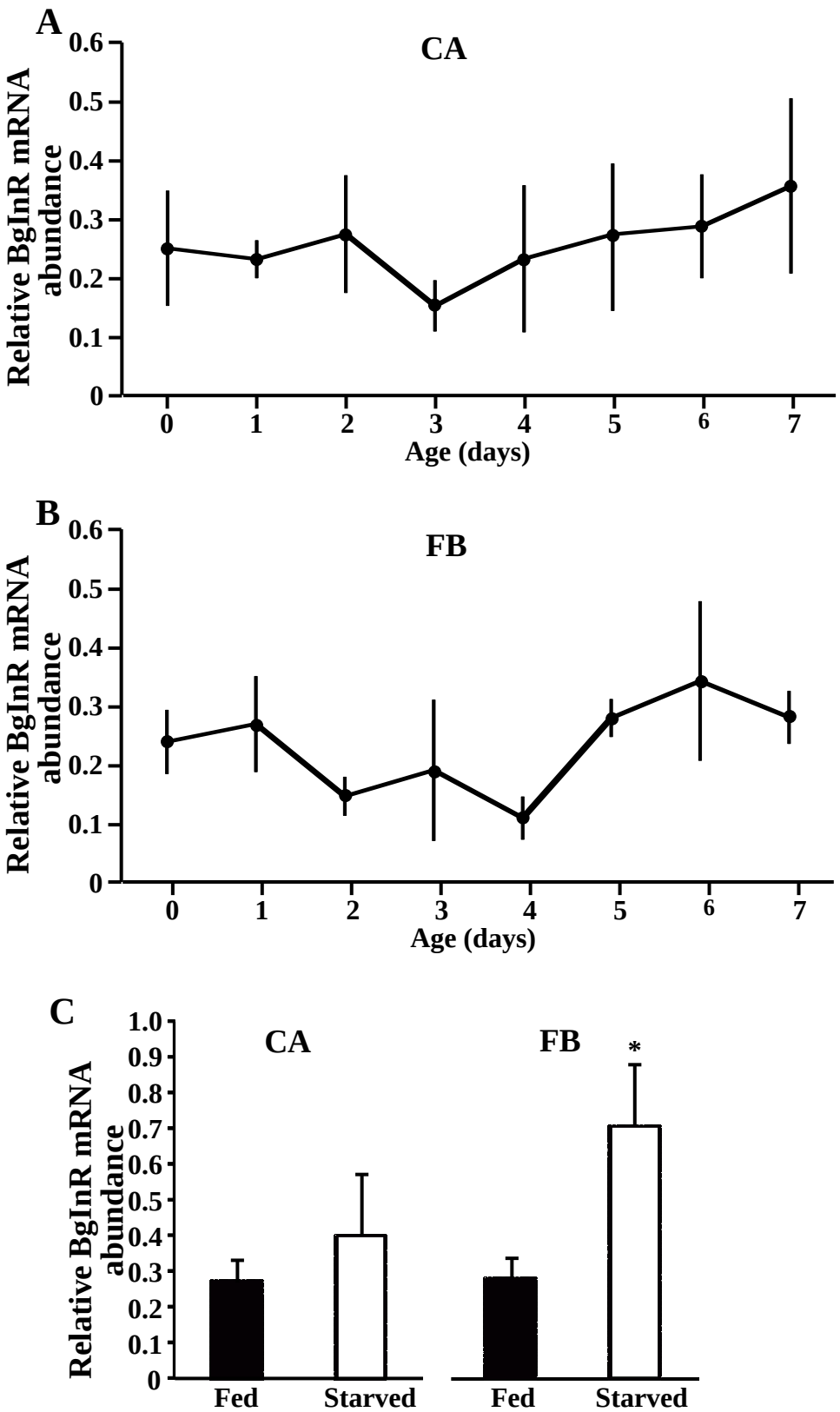

A
CA
Relative BgInR mRNA abundance
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7
Age (days)
B
FB
Relative BgInR mRNA abundance
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7
Age (days)
C
CA
FB
*
Relative BgInR mRNA abundance
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fed
Starved
Fed
Starved

Figure 3

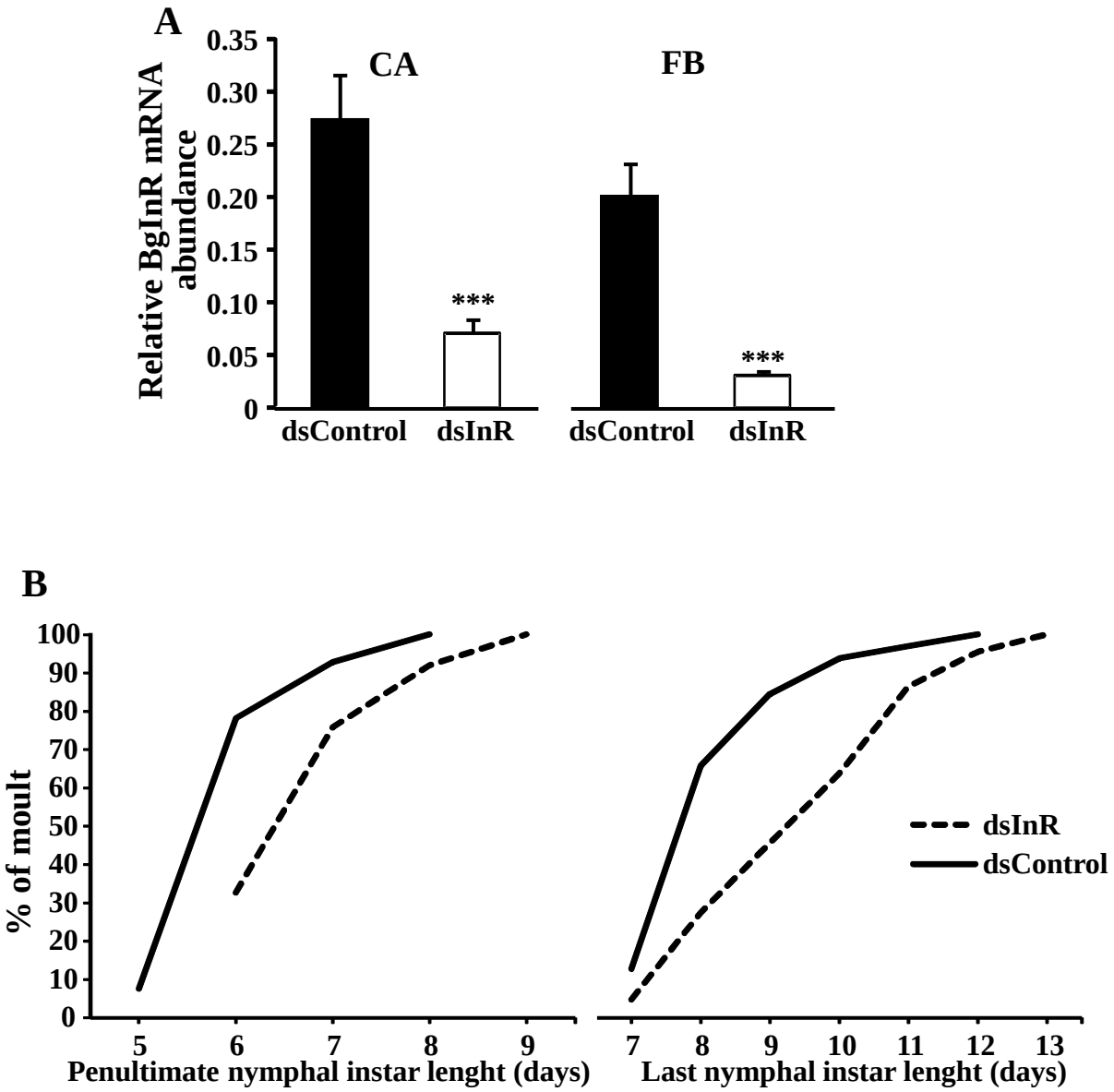

A
Relative BgInR mRNA abundance
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
CA
FB
***
***
dsControl
dsInR
dsControl
dsInR
B
% of moult
100
90
80
70
60
50
40
30
20
10
0
5
6
7
8
9
Penultimate nymphal instar lenght (days)
7
8
9
10
11
12
13
Last nymphal instar lenght (days)
dsInR
dsControl

Figure 4

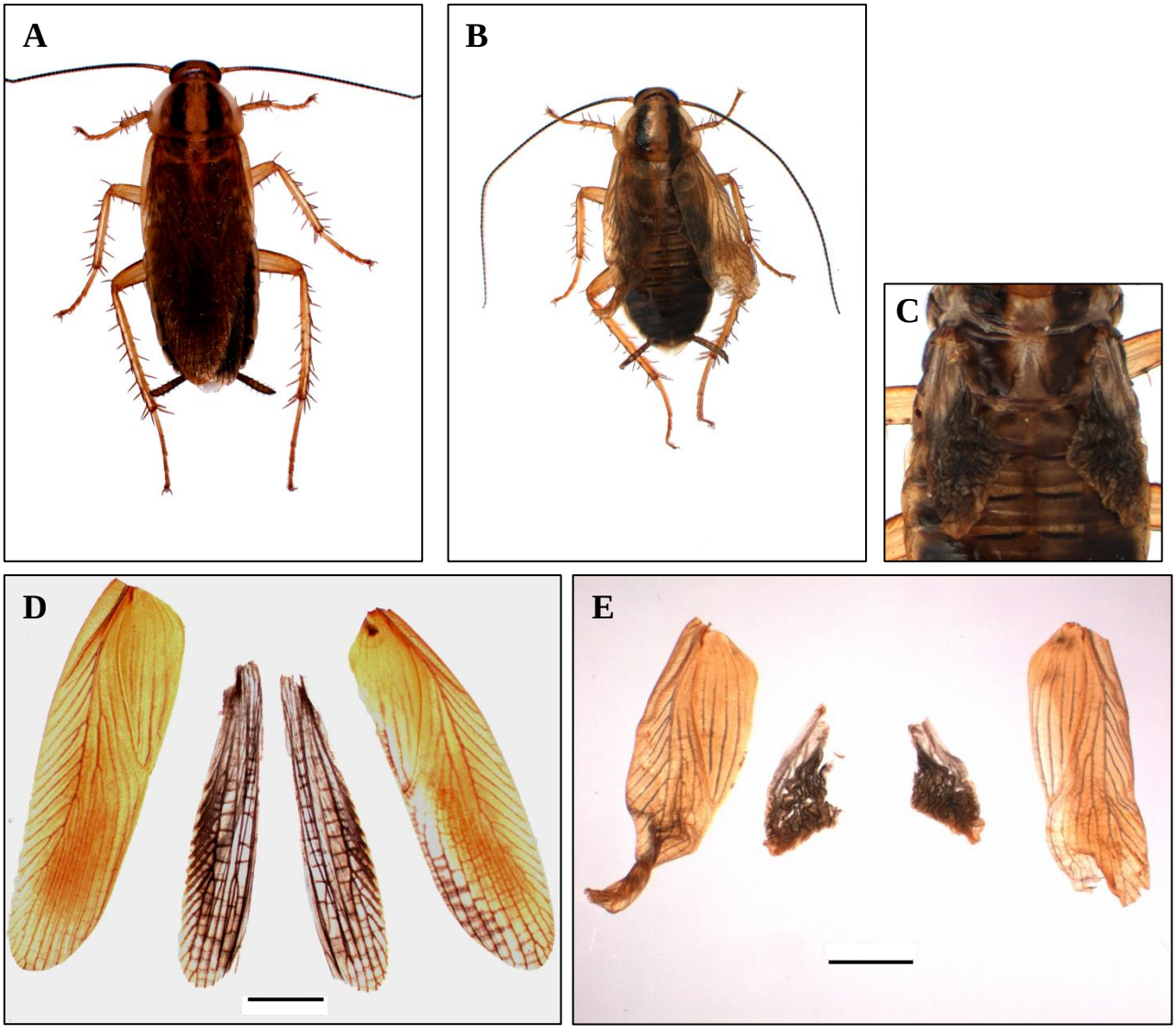


Figure 5

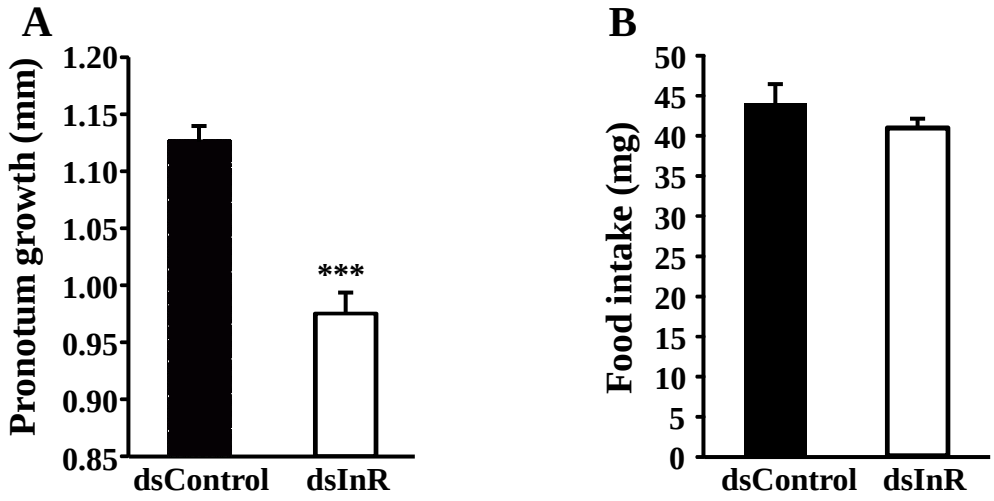

A
Pronotum growth (mm)
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
***
dsControl
dsInR
B
Food intake (mg)
50
45
40
35
30
25
20
15
10
5
0
dsControl
dsInR

Figure 6

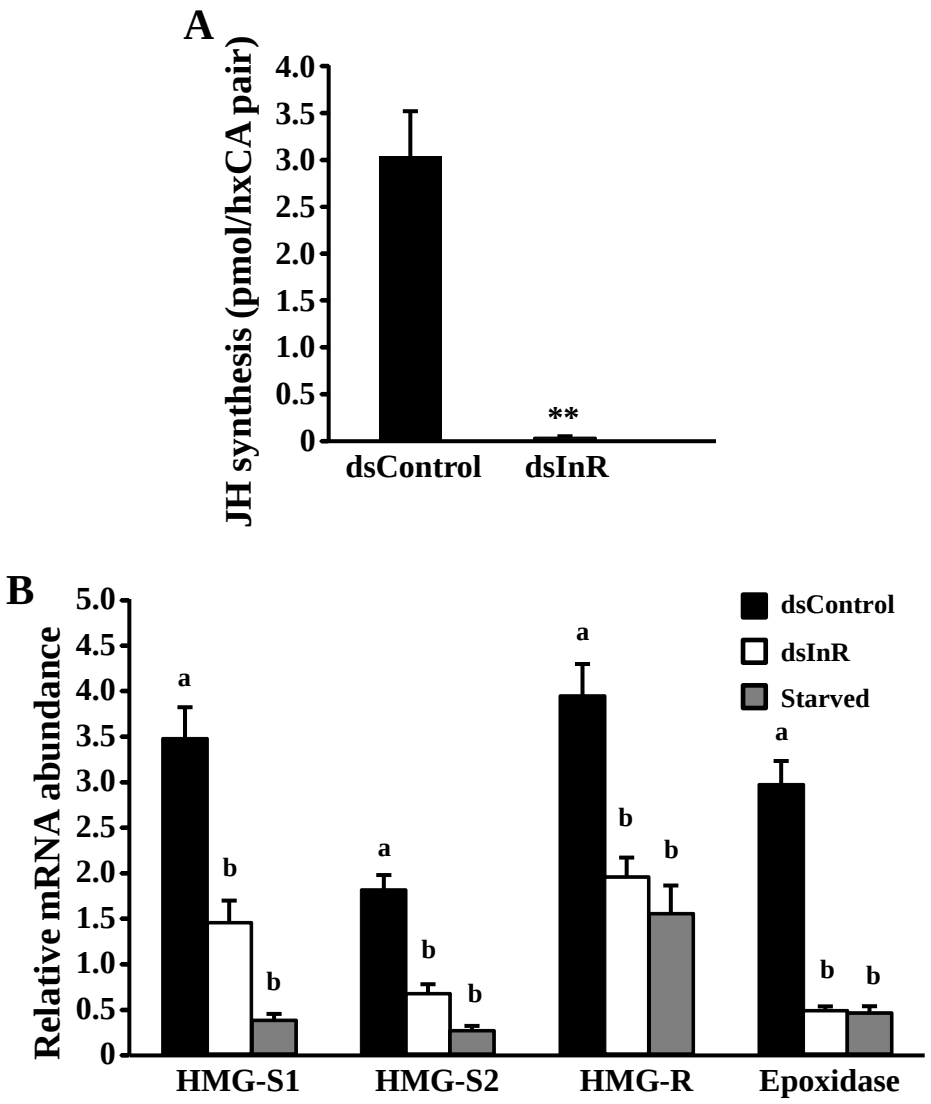

A
JH synthesis (pmol/hxCA pair)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
**
dsControl
dsInR
B
Relative mRNA abundance
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
dsControl
dsInR
Starved
a
b
b
a
b
b
a
b
b
a
b
b
HMG-S1
HMG-S2
HMG-R
Epoxidase

Figure 7

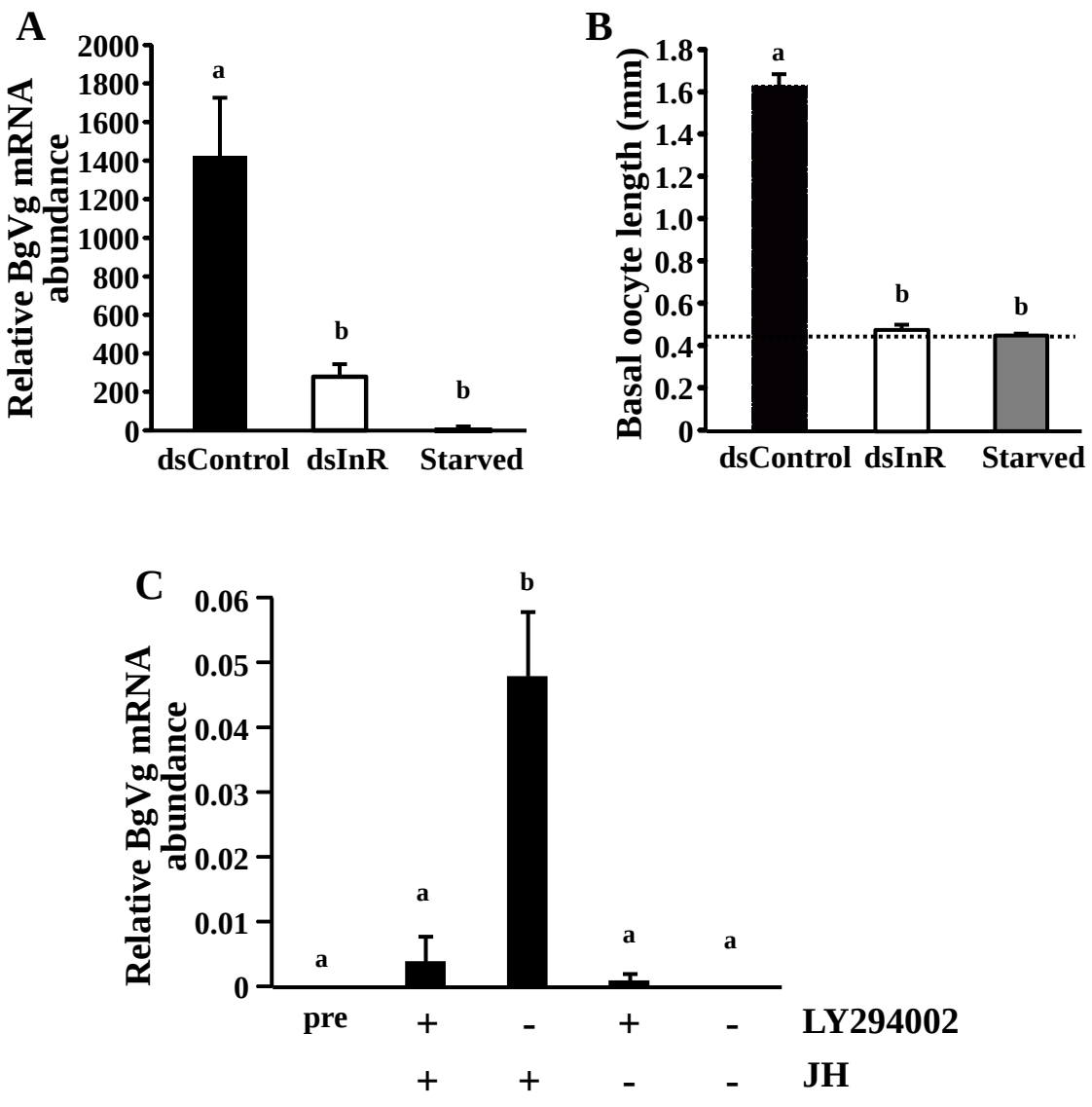

A
Relative BgVg mRNA abundance
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
b
dsControl
dsInR
Starved
B
Basal oocyte length (mm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
a
b
b
dsControl
dsInR
Starved
C
Relative BgVg mRNA abundance
0.06
0.05
0.04
0.03
0.02
0.01
0
a
a
b
a
a
pre
+
-
+
-
LY294002
+
+
-
-
JH

Figure 8

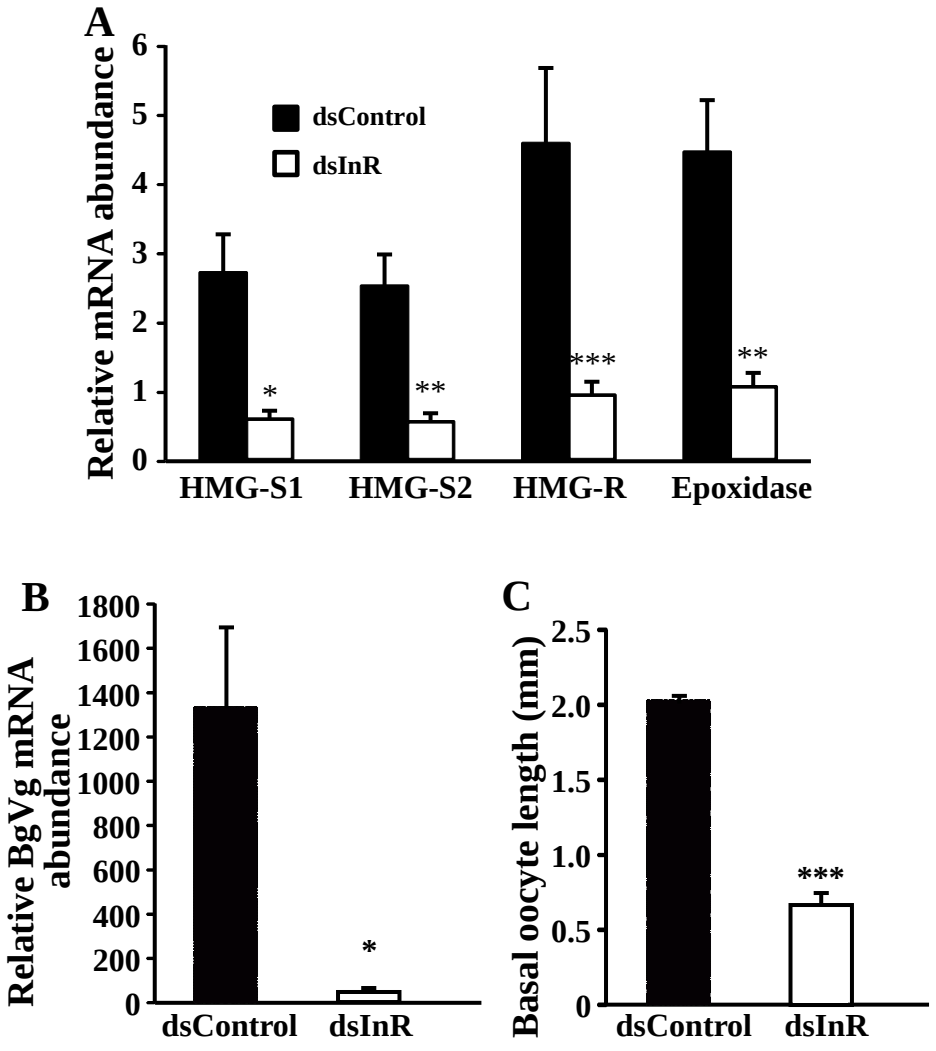
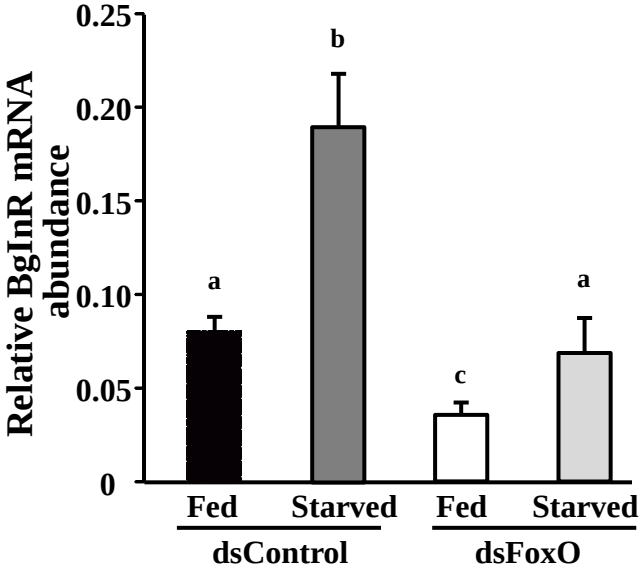
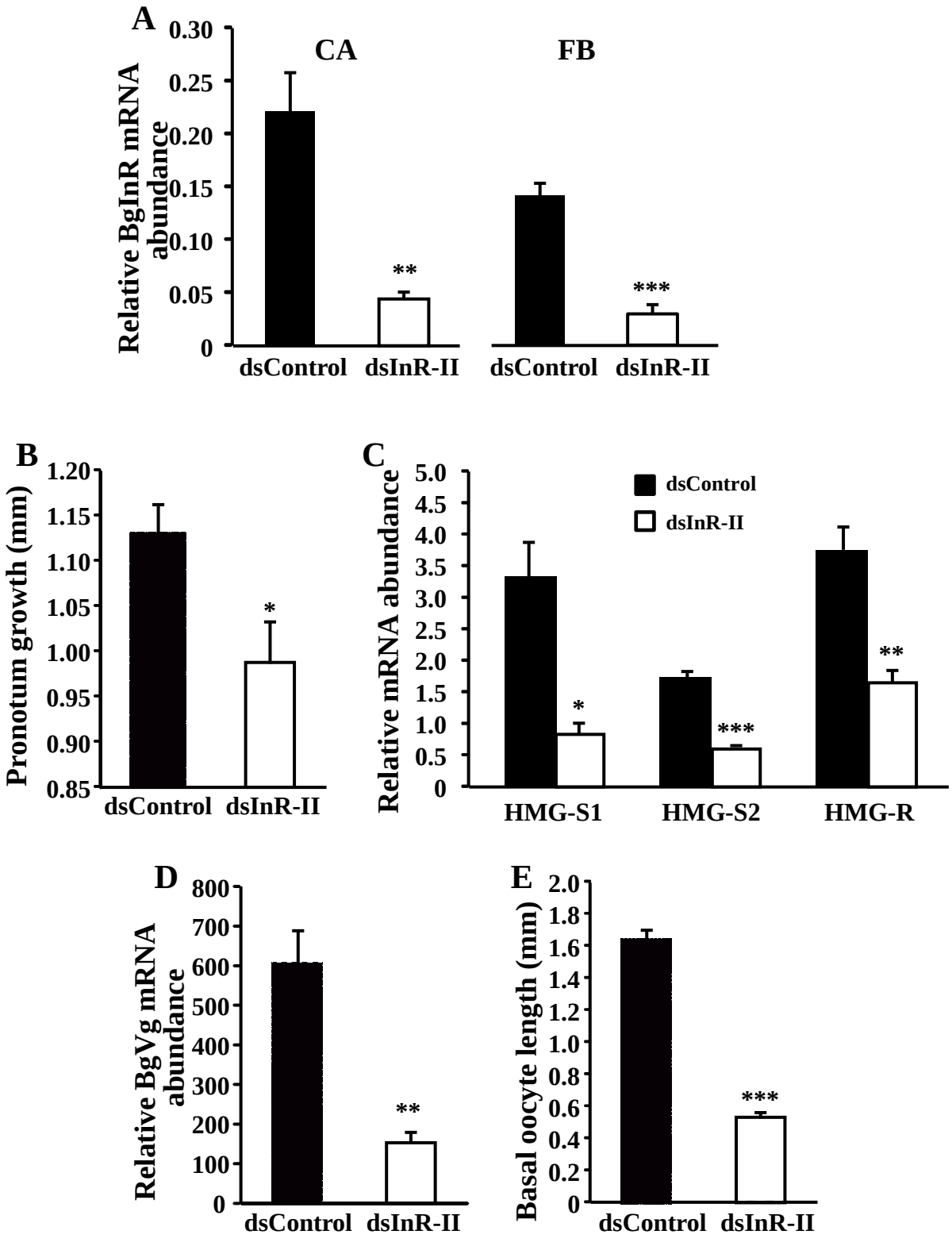

A
Relative mRNA abundance
6
5
4
3
2
1
0
dsControl
dsInR
*
**
***
**
HMG-S1
HMG-S2
HMG-R
Epoxidase
B
Relative BgVg mRNA abundance
1800
1600
1400
1200
1000
800
600
400
200
0
*
dsControl
dsInR
C
Basal oocyte length (mm)
2.5
2.0
1.5
1.0
0.5
0
***
dsControl
dsInR

Figure 9


Relative BgInR mRNA abundance
0.25
0.20
0.15
0.10
0.05
0
a
b
c
a
Fed
Starved
Fed
Starved
dsControl
dsFoxO

Supplementary Figure



Supplementary Fig. 1. Effect of BgInR RNAi (dsFoxO II) on CA and fat body. dsRNA targeting BgInR (*dsInR-II*) or a non-homologous dsRNA (*dsControl*) was administered on the first day of the penultimate (fifth) and last (sixth) nymph instars. Dissections were performed five days after the adult moult. (A) BgInR mRNA levels in CA (n=7-9) and fat body (n=7-10). (B) Pronotum growth during the penultimate and last nymphal instars (n=5-8). (C) mRNA levels of HMG-CoA synthase-1, -2 and HMG-CoA reductase (n=7). (D) BgVg mRNA levels in fat bodies (n=7). (E) Basal oocyte lengths (n=4). In (A), (C) and (D), Y-axis indicates copies per copy of BgActin 5C. Results are expressed as the mean ± S.E. Asterisks represent significant differences (Student's *t* test, **P*<0.05; ***P*<0.001; ****P*<0.0001).

Table 1. Primer sequences.

Degenerated primers used for BgInR cloning

Forward	5′-TT(C/T)GGNATGGTNTA(C/T)(A/G)A(A/G)GG-3′
Reverse-1	5′-TNGGNGA(T/C)TT(T/C)GGNATG(A/G)C-3′
Reverse-2	5′-GCNGCN(C/A)GNAA(T/C)TG(T/C)ATG-3′

Primers used for synthesizing dsInR and dsInR-II

Forward dsInR	5′-ATGCAACAGATCGTGAGAGAAGTG-3′
Reverse dsInR	5′-ATCTTCAGCAACCATGCAATTCC-3′
Forward dsInR-II	5′-ACAACGAATTGCTCTCAGCAAAGTT-3′
Reverse dsInR-II	5′-AGCGTACAGCTGTGTAATCTCCAA-3′

Primers used for qPCR

Forward BgInR	5′-CACAGGGCCTAATTCCACAGA-3′
Reverse BgInR	5′-ACAGCGCCGGTTCAGATACTT-3′