

Insulin receptor substrate influences female caste development in honeybees

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The insulin/insulin-like signalling (IIS) network is conserved among animals and is central to growth and development. In eusocial honeybees (*Apis mellifera*), IIS is hypothesized to shape female caste fate. We tested this hypothesis via RNA interference (RNAi) knockdown of the insulin receptor substrate (IRS) homologue, a key adaptor protein in IIS. Female larvae naturally develop into queens (reproductives) or workers (helpers) after being fed rich versus limited diets, respectively. Feeding larvae a rich diet mixed with dsRNA (double stranded RNA) targeting *irs* gene transcript decreased *irs* mRNA abundance and caused development of worker morphology. Controls receiving rich larval diet and control dsRNA developed queen morphology. Whole-body mass spectrometry profiling of larvae collected 72, 96 and 120 h after dsRNA treatments revealed proteomic differences between *irs* gene knockdowns and controls, including levels of hexamerin 110, a storage protein connected to natural caste differences.

Keywords: insulin signalling; *Apis mellifera*; queen bee; worker bee; RNA interference; proteomics

1. INTRODUCTION

Female honeybees develop into two castes, queens and workers, which differ in morphology, physiology, social function and longevity. The reproductive queens lay up to 2000 eggs daily, develop into adults faster, are bigger, heavier, have larger reproductive organs and survive longer (more than 1.5–2 years) than workers [1]. Workers live for two weeks to several months and are mostly sterile helpers that nourish larvae and collect food. The queen–worker diphenism is believed to be central to the ecological success of honeybees, and is mediated socially by larval feeding: high quantity and quality of larval provisions result in queens while more limited nourishment yields workers, independent of genetic background [1]. On the molecular level, juvenile hormone, DNA methylation and TOR (target of rapamycin), a protein kinase involved in nutrient-sensing and growth control, have been independently shown to influence caste development [2,3].

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rsbl.2010.0463> or via <http://rsbl.royalsocietypublishing.org>.

Received 17 May 2010

Accepted 14 June 2010

Insulin-like signalling (IIS), which can be coupled to TOR activation through downstream integration of Tsc-1 and 2 (tuberous sclerosis complex genes 1 and 2; e.g. [4]), is similarly crucial to nutrient-sensing and growth regulation in animals. IIS influences development and final body size, food-related behaviour, reproduction and longevity across many taxa ([5] and references therein). Thus, this pathway is also likely to influence honeybee caste development [6,7]. However, functional evidence for this hypothesis has been limited.

Most of what has been revealed about IIS in animal model systems relies on impaired insulin signalling caused by either knockout (disruptive mutation) or knockdown (downregulation via RNA interference, RNAi) of key insulin signalling proteins like the insulin receptor substrate (IRS) protein. Downregulation of IRS confers a substantial reduction of IIS [8]. Recently, we established RNAi-mediated *irs* gene knockdown in adult honeybee workers to show that the IRS product influences food-choice behaviour towards pollen (amino acids) and nectar (carbohydrate) [9].

Downregulation of IIS via disruptive mutation of *irs* reduces body size and fertility in *Drosophila* [10]. Thus, we reasoned that the course of larval development in response to rich queen diet could be blocked by decreasing IIS through *irs* repression. This should yield the small, essentially sterile worker phenotype, as previously shown, following downregulation of TOR gene transcript [3]. To test this hypothesis, we suppressed *irs* expression by RNAi in larvae reared on laboratory diet that elicits queen development. This study provides functional evidence for IIS roles in honeybee caste development.

2. MATERIAL AND METHODS

(a) *irs* dsRNA synthesis, bees, feeding treatments, sample collections

Synthesis of *irs* dsRNA was conducted as before [9]. Larvae were produced from two wild-type (and multiply mated) queens and genotype was a random factor in the experiment. *In vitro* feeding methods were essentially identical to those of [3]. We produced two treatment groups: larvae-fed queen diet mixed with *irs* dsRNA, and larvae-fed queen diet mixed with a standard control of *gfp* (green fluorescent protein) dsRNA.

Two replicate studies with 100 larvae per treatment group were performed. A random subset of larvae was collected into liquid nitrogen 72, 96 and 120 h after the first dsRNA feeding (see the electronic supplementary material).

(b) RNAi validation, morphological phenotyping

irs RNA abundance was tested by quantitative real-time PCR 72 h after dsRNA feeding began (see the electronic supplementary material). Developmental time (larva to adult) was assessed ($n = 50$), and a random subset ($n = 20$) measured at adult emergence for fresh weight. The first 12 individuals (only queens in the control group as intercastes typically emerge later) were dissected to determine ovary size by counting ovarian filaments (ovarioles) as before [3].

(c) Protein extraction, tryptic digestion and proteomics analysis

These steps (see the electronic supplementary material) were essentially carried out as before [11]. Proteomics analysis was conducted using liquid chromatography coupled to tandem mass spectrometry (LC-MS²).

(d) Statistical analysis

Non-parametric Mann Whitney *U*-tests were used to individually compare *irs* expression, fresh weight, developmental time and ovary size between groups. Proteomic data were analysed by an established combination of Mann Whitney *U*-tests with correction for type 1 error inflation (see the electronic supplementary material; [12]).

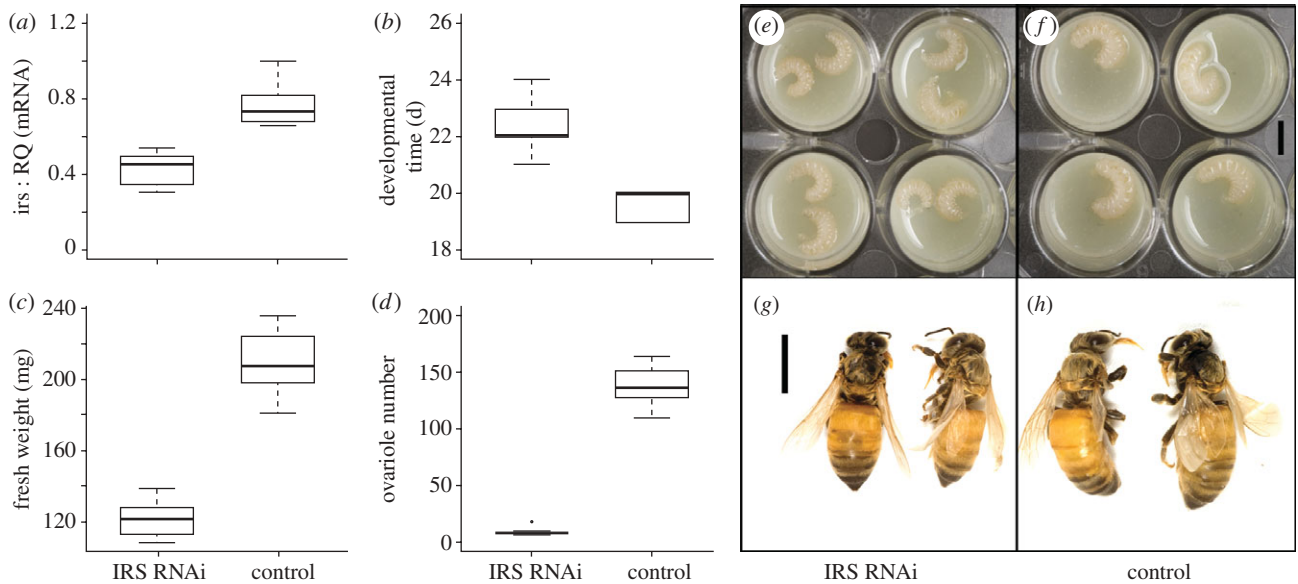


Figure 1. Physiological characters differ between *irs* knockdowns and control honeybees. (a) *irs* expression, (b) developmental time, (c) fresh weight and (d) ovariole number. Boxplots show medians and 25–75 percentiles. All traits diverged between groups (Mann Whitney *U*-tests, $p < 7.4E - 07$). (e,f) Images of representative individuals in early 5th larval instar and (g,h) as newly emerged adults visualize the phenotypic differences (scale bar, 0.5 cm).

3. RESULTS

(a) Verification of *irs* RNAi

Larvae treated with *irs* dsRNA in queen diet showed reduced whole-body *irs* expression compared with control larvae-fed queen diet with *gfp* dsRNA ($p < 7.4E - 07$, $n = 12$, figure 1a). RNA quantification to *actin* or *tubulin* housekeeper genes gave corresponding results (see the electronic supplementary material).

(b) Morphological phenotyping

Compared with controls, *irs* knockdowns took longer to develop ($p < 2.2E - 16$, $n = 50$, figure 1b), had lower fresh weight ($p < 1.5E - 11$, $n = 20$, figure 1c), fewer ovarioles ($p < 7.4E - 07$, $n = 12$, figure 1d), and were smaller in size throughout development (figure 1e–h). As adults ($n = 50$), more than 80 per cent *irs* knockdowns exhibited a worker-specific structure on their hind leg (corbicula, used to collect pollen) and lacked the mandibular notch typical of queens. The residual individuals (less than 20%) had one intercaste character; either lack of corbicula or presence of mandibular notch. As previously noted [3], more than 50 per cent of controls had queen morphology, while the remaining individuals (less than 50%) displayed one intercaste trait (presence of corbicula or lack of mandibular notch, total $n = 30$). Queen versus worker caste frequencies were significantly different between groups (Chi-square = 55.0, d.f. = 1, $p < 1.0E - 5$).

(c) Proteotyping

LC-MS/MS-based proteomics was conducted on larvae collected 72, 96 and 120 h after the first feeding of dsRNA. A conservative data analysis corrected for an over-abundance of royal jelly proteins in the sample material suggested diverse metabolic differences between knockdown larvae and the control

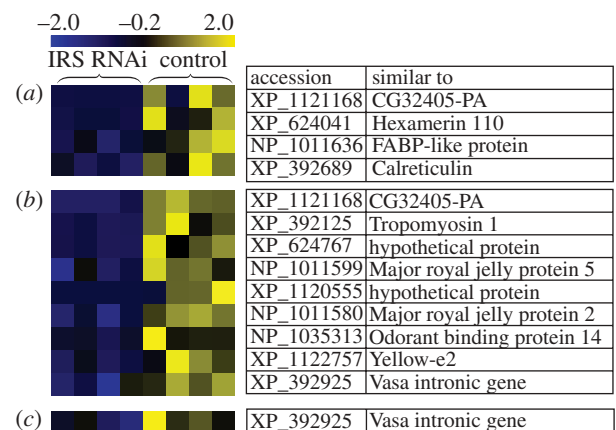


Figure 2. Proteomic profiling reveals larval proteins affected by the *irs* knockdown. Proteomic analysis detected significant differences (a) 72, (b) 96 and (c) 120 h after treatments with dsRNA began (Mann Whitney *U*-tests, $p < 0.05$); visualized in a heatmap. Columns represent replicates and rows individual proteins. Values range from low (blue) to high (yellow). Only proteins with higher abundance in controls are shown to curtail type 1 error (see §4 for details).

group (Mann Whitney *U*-tests, $p < 0.05$, $n = 4$, figure 2) including hexamerin 110, a fatty-acid binding protein and the product of *vasa intronic gene* (VIG). All proteomics data have been deposited in NCBI's Peptidome database (<http://www.ncbi.nlm.nih.gov/peptidome/repository/PSE129>).

4. DISCUSSION

It was hypothesized that IIS plays a central role during honeybee caste development, but functional evidence for the idea was largely missing. We fill this void by establishing that honeybee queen development is

blocked by RNAi-mediated repression of *irs*, encoding a central player in IIS. We observed longer developmental times, reduced fresh weights, smaller ovaries and presence of corbicula but absence of mandibular notch in *irs* knockdowns. These findings are in good agreement with characteristic differences between queen and worker bees reared in natural nests [1].

Our study complements that of Patel *et al.* [3], who reported the same developmental effect of reducing nutrient sensing by TOR RNAi. Taken together, these results show that honeybee queen development cannot proceed if IIS or TOR transduction is reduced.

We profiled the proteome of larvae to observe the molecular response to *irs* RNAi. As a conservative measure, we opted to exclude proteins with reduced abundance in controls because royal jelly proteins were more abundant in this group. Honeybee larvae are submerged in jelly protein, and differences in amounts of jelly carried over to the proteomic analysis (e.g. owing to different larval size or gut content of food), could lead to underestimation of some relative protein levels in controls. Our analysis excluded these (putative) false positives.

Hexamerin 110 was reduced in *irs* knockdown (figure 2). Hexamerin protein levels were previously reported to be lower in worker-destined than in queen-destined larvae of *Apis mellifera* and other social Hymenoptera, e.g. *Polistes metricus* [13,14]. Furthermore, a link between hexamerin and caste is found in termites, where hexamerin RNAi biases development toward soldiers and away from reproductives [15]. The hexamerin protein family, therefore, can be central to caste differentiation in social insects, perhaps by representing a caste-specific nutrient storage and supply [16].

Higher levels of a fatty acid binding protein (FABP-like) homologue in controls suggested that fatty acid metabolism was altered by *irs* RNAi. In vertebrates, FABPs may have indirect effects on IIS with lower levels conferring increased insulin sensitivity [17]. A putative connection of IIS to the metabolism of fatty acids can now be explored in honeybees.

The difference in CG32405-PA (annotated as cuticular protein 65Acv) levels suggests diverging cuticular profiles in larvae of the two castes, which could be connected to caste recognition.

VIG (the product of the vasa introgenic gene) associates with microRNAs and the RNA-induced silencing complex (RISC) of the *Drosophila* RNAi machinery [18]. These features make VIG a possible master regulator of gene expression. Our finding that a VIG encoding gene homologue was differently expressed between *irs* knockdowns and controls supports the proposition that differential microRNA regulation plays an important role in honeybee caste differentiation [19].

In summary, we show here that honeybee queen development requires IIS transduction. Our protein profiling confirms physiological changes within hours following *irs* downregulation, lending support to the hypothesis that honeybee caste development relies on IRS-dependent mechanisms. Our findings bring us closer to solving the puzzle of honeybee caste

development and exemplify how ancient gene networks can be co-opted during caste evolution in social insects.

This work was supported by the PEW Foundation, the Alexander-von-Humboldt Foundation, the Norwegian Research Council (180504, 185306, 191699), National Institute of Ageing (PO1AG22500), and the Binational Science Foundation (2007465). Additional acknowledgements in the electronic supplementary material.

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