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RESEARCH ARTICLE

Insulin-like peptides (AmILP1 and AmILP2) differentially affect female caste development in the honey bee (*Apis mellifera* L.)

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SUMMARY

The food a honey bee female larva receives determines whether she develops into a large long-lived fertile queen or a short-lived sterile worker. Through well-established nutrient-sensing and growth-promoting functions in metazoans, the insulin/insulin-like growth factor 1 signaling (IIS) pathway has become a focal topic in investigations on how differences in food environment can be translated into internal signals responsible for queen–worker determination. However, low expression levels of two insulin receptors (AmInRs) in honey bee larvae and the failure of one AmInR to influence caste differentiation are in potential conflict with such a classical growth-promoting role of IIS in queen–worker development. In view of such an apparent contradiction, and the fact that binding partners and affinities of these two AmInRs have not been worked out, we performed a functional study on insulin-like peptide genes (*AmILP1* and *AmILP2*) in honey bee larvae by using a double-stranded RNA (dsRNA)-mediated gene knockdown approach. We found that juvenile hormone (JH) levels were diminished by *AmILP1* dsRNA treatment, while the *AmILP2* knockdown caused a reduction in ovary size. Blood sugar titers were not significantly affected by the treatments. From these results we conclude that *AmILP2* transcript levels may influence specific organ development, such as the ovary and body mass, while more general traits of caste differentiation, such as mandibles, may require additional regulators. In addition, JH production may be regulated by *AmILP1* expressed locally in the brain, similar to the function of certain *ILPs* in *Drosophila*.

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INTRODUCTION

Eusocial insects, including honey bees, exhibit an environmentally induced caste polyphenism that promotes colony efficiency through a morphology-based division of labor: while queens are functional egg-laying machines, workers forgo reproduction and, instead, care for the brood, defend the colony and forage for food (Hölldobler and Wilson, 2008). The ecological and evolutionary success of social insects (Hölldobler and Wilson, 2008) is largely built upon such division of labor, but the mechanisms that generate alternative phenotypes (castes) in Hymenoptera and termites are not fully understood (Hartfelder and Emlen, 2012).

The most considerable progress in understanding caste development has been made in the honey bee, *Apis mellifera* L., where the primary trigger is differential feeding of the larvae. Queen larvae receive copious amounts of royal jelly, a glandular secretion produced by young worker bees, throughout all five larval instars (Haydak, 1970; Winston, 1987). In contrast, worker larvae are fed less frequently and receive a diet less rich in sugar (4% compared with 12% in royal jelly) during the third and fourth larval instars (Asencot and Lensky, 1985). These diets induce a series of endogenous responses that result in differential phenotypes. The most studied endocrine regulator of caste differentiation is juvenile hormone (JH) (Rachinsky et al., 1990; Rembold, 1987), which shows higher titers during the fourth to fifth instar in queen-destined larvae (Rachinsky and Hartfelder, 1990; Rachinsky et al., 1990). Functionally, JH application induces queen-like traits in larvae with a restricted diet (Goewie, 1977; Rembold et al., 1974). Although the mode of action of JH in driving queen development is still rather unclear, its effect on ovary size (i.e. ovariole number), which is one of the key morphological traits differing between queens and workers, has been revealed. JH affects ovary differentiation from the third larval instar until the onset of metamorphosis: high JH titers in queen larvae prevent autophagic programmed cell death in the ovary (Schmidt Capella and Hartfelder, 2002), thus sustaining tissue survival and differentiation into the large queen ovaries, whereas low JH titers in worker larvae cannot inhibit programmed cell death, which removes 95–99% of the ovariole primordia and leads to the small worker-type ovaries.

Ovary size defines the reproductive status of queens and workers (Winston, 1987), and regulates worker social behaviors (Wang et al., 2009; Wang et al., 2010). The adult honey bee queen has up to 150 ovarioles in each of her ovaries and is only responsible for laying eggs. In contrast, workers, which are functionally sterile, typically only have on average 2–12 ovarioles per ovary (van der Blom et al., 1994; Michener, 2000; Winston, 1987). However, the ovariole numbers may vary, and *in vitro* rearing experiments showed that there is a morphospace gradient in which ovary phenotypes of the queen and worker are the extremes (Leimar et al., 2012; Linksvayer

et al., 2011). In addition, ovary size is also correlated with foraging behavior in workers: workers with more ovarioles perform less retinue behavior (D. Galbraith, Y.W., G.V.A., R. E. Page and C. G. Grozinger, unpublished data), initiate foraging tasks earlier in life (Wang et al., 2009; Wang et al., 2010), prefer to collect pollen over nectar, and are more sensitive to sucrose than workers with fewer ovarioles (Amdam et al., 2006; Wang et al., 2009).

The release of the honey bee genome sequence (Honeybee Genome Sequencing Consortium, 2006) greatly facilitated investigations on how the nutrient stimuli are translated into endogenous molecular signals in honey bee caste differentiation. The focus has been on two conserved eukaryotic nutrient-sensing pathways: the insulin/insulin-like growth factor 1 signaling (IIS) pathway (Mutti et al., 2011a; Wolschin et al., 2011) and the closely related and interacting target-of-rapamycin (TOR) pathway (Patel et al., 2007). Larvae subjected to RNA interference (RNAi)-mediated gene knockdown of the insulin receptor substrate (*IRS*) and *TOR* genes consistently developed into workers even when receiving a queen diet (Kamakura, 2011; Mutti et al., 2011a; Patel et al., 2007). As RNAi primarily targets the fat body (Jarosch and Moritz, 2011), a tissue functionally homologous to white adipose tissue and the liver in mammals (Chapman, 1998), these studies also provided evidence that *IRS* and *TOR* genes expressed in the fat body may remotely control JH production by the corpora allata (CA) in the retrocerebral complex (Mutti et al., 2011a). In addition, gene expression studies have revealed that the genes encoding two insulin-like peptides (*AmILP1* and *AmILP2*) and two insulin receptors (*AmInR1* and *AmInR2*) are differentially expressed between queen and worker larvae (de Azevedo and Hartfelder, 2008; Wheeler et al., 2006). Together with the results of a recent study showing that epidermal growth factor receptor (*EGFR*) gene knockdown induces the worker phenotype (Kamakura, 2011), the current evidence indicates that caste development in honey bees involves a complex interaction network composed of the IIS/TOR/EGFR pathways, JH and ecdysteroids, which are classic developmental and reproductive hormones in *Drosophila* (Mirth and Riddiford, 2007) and other insect species (Chapman, 1998).

Nonetheless, upon a closer look, the regulatory network of honey bee caste development is not straightforward, especially for the IIS pathway. For instance, the expression levels of *AmInR1* and *AmInR2* in fourth instar queen larvae decline to very low levels, just as the larvae show the highest growth rates (de Azevedo and Hartfelder, 2008). Furthermore, silencing one of the *AmInR* genes did not affect caste fate in honey bees (Kamakura, 2011), suggesting the effect of IRS on queen–worker phenotype differentiation may be mediated by EGFR, and not through IIS (Mutti et al., 2011a).

Clearly, based on sequence similarity, *AmInR1* and *AmInR2* are putative genes for receptors of insulin-like peptides, the upstream signaling factors in IIS. However, their binding partners and respective binding affinities have not been investigated. The honey bee *AmILP1* and *AmILP2* genes also have high sequence similarity to *Drosophila ILPs* (*DILPs*) whose roles in the IIS have been intensively investigated. Previous studies on *AmILP1* and *AmILP2* in honey bee brain and fat body suggested that the proteins encoded by *AmILP1* and *AmILP2* genes mediate nutritional signals (Ament et al., 2008; Ament et al., 2010) and regulate energy metabolism (Wang et al., 2012), which are conserved functions of ILPs across species including *Drosophila*. Studies on *AmILP1* levels in the brain of honey bee workers (Ament et al., 2010; Corona et al., 2007) have shown that these are negatively correlated with individual nutritional status and positively related to JH titer (Ament et al., 2008). Additionally, low levels of *AmILP1* transcripts in the fat body of adult bees are linked to high blood sugar levels (Wang et al., 2012). In contrast, the regulation and function of AmILP2 is less well understood as *AmILP2* expression does not consistently respond to factors as *AmILP1* does in adult honey bees (Amdam, 2011; Wheeler et al., 2006).

In *Drosophila*, silencing *DILPs* strongly affected larval development and carbohydrate metabolism (Brogiolo et al., 2001; Rulifson et al., 2002). Although gene expression profiles of *AmILPs* in honey bee larvae differ among the castes (de Azevedo and Hartfelder, 2008; Wheeler et al., 2006), actual functional data of *AmILP1* and *AmILP2* in larval development are still missing. Thus, to gain insight into *AmILP1* and *AmILP2* gene function in queen–worker differentiation we used an RNAi-mediated gene knockdown approach in larvae reared in an *in vitro* system (Patel et al., 2007). The treated and control larvae were assayed for transcript levels of *AmILP1* and *AmILP2*, hemolymph sugar and JH levels, and larval body mass at the fifth larval instar, when developmental hormone titers (JH and ecdysteroids) are very different and when the caste-specific differentiation of the ovaries is in the most pronounced stage. In addition, we screened the expression of caste phenotype characters of the adults that emerged from such treatments. The results are indicative of differential roles for the *AmILPs* in the queen–worker differentiation process.

MATERIALS AND METHODS

Experimental design

In this study, we used a full factorial design in which *AmILP1* RNAi and *AmILP2* RNAi treatments are the two independent factors. There were two levels for each of the factors: ‘0’ (no RNAi) and ‘1’ (RNAi). It is known that a factorial experimental design is ‘more efficient than one-factor-at-a-time experiments and can detect interactions’ (Montgomery, 1997). This allowed us to study the effect of each factor on the traits we are most interested in, as well as the effects of interactions between the two factors on those traits (Montgomery, 1997). Studies on ILPs in other insects have found that the functions of ILPs are usually linked (Wu and Brown, 2006). And it has been proposed that AmILP1 and AmILP2 acts as agonist and antagonist of their respective receptors, but no experimental evidence has been found so far (Nilsen et al., 2011). Therefore, determining the interaction between *AmILP1* and *AmILP2* should be informative for understanding how the functions of *AmILP1* and *AmILP2* are interconnected in honey bees.

Double-stranded RNA synthesis

DNA fragments of the *AmILP1* and *AmILP2* genes flanked on both sides with a T7 promoter sequence were inserted into the commercial T-easy vector (Promega, Madison, WI, USA) using the primers listed in supplementary material Table S1. Plasmids were extracted and sequenced to validate the DNA sequences of *AmILP1* and *AmILP2*. Double-stranded RNAs (dsRNAs) of *AmILP1* and *AmILP2* were synthesized following a previously established protocol (Amdam et al., 2003). The gene sequence of the green fluorescent protein (GFP), which is not found in the honey bee genome, was used to produce a non-target dsRNA, serving as a control dsRNA in the RNAi assays.

In vitro rearing of honey bee larvae

Wild-type honey bees maintained at the Honeybee Research Facility at the Arizona State University Polytechnic campus (Mesa, AZ, USA) were used in these experiments. Queens from three wild-type colonies were caged for 24 h and newly hatched larvae (12–18 h old, N=1000) were grafted into Petri dishes containing a previously

established nutrient-rich diet suitable for *in vitro* rearing of queens (Patel et al., 2007), and were kept in a cell culture incubator at 33°C and 80% humidity (Patel et al., 2007). On the second day, larvae of similar size were grafted from the Petri dishes and randomly distributed into 24-well culture plates (6 larvae per well). A full factorial design was applied on our dsRNA feeding regime, and *AmILP1* dsRNA and *AmILP2* dsRNA were used as two independent factors. Four experimental treatments were created: *AmILP1* dsRNA, *AmILP2* dsRNA, *AmILP1+AmILP2* dsRNAs, and *gfp* dsRNA. Each well contained the larval diet supplemented with 200 μg ml^{-1} of each respective dsRNA. Therefore, the total dsRNA was 200 μg ml^{-1} for *AmILP1* dsRNA, *AmILP2* dsRNA and *gfp* dsRNA treatment groups, and 400 μg ml^{-1} for the *AmILP1+AmILP2* dsRNA group. Using similar factorial designs in both honey bee larvae and adults, previous studies on gene knockdown have shown that the amount of dsRNA in combined treatment groups does not cause any unspecific or adverse effects (Mutti et al., 2011a; Wang et al., 2012). Every 12 h, the larvae were transferred to new diets in new plates, with changes of the position on the plate in a randomized design to minimize any location effects. After 2 days of feeding on the dsRNA-containing diet and 1.5 days of feeding on dsRNA-free diet, 20 larvae from each treatment group were collected to validate the gene knockdown and to reveal larval physiology responding to the treatments. The remaining larvae continued to be fed with dsRNA-free diet until they began defecating. Subsequently, they were transferred to filter paper-lined Petri dishes, with the filter paper being changed every day as they passed the pupal stage, and finally emerged as adult bees in the Petri dishes.

Sampling of hemolymph and larval body for gene expression, blood sugar and JH level analyses

Larvae retrieved from the experimental setup were cleaned by carefully wiping with tissue paper, and were weighed on a digital scale (VWR, Gaithersburg, MD, USA). The body of the larva was pierced with a 30 gauge BD needle, so that two samples of extruding hemolymph could be collected from each larva with glass capillaries (VWR). These hemolymph samples were used to assay sugar levels and JH titers, respectively. The hemolymph samples for carbohydrate measurement were immediately frozen on dry ice and kept at −80°C until use. The hemolymph samples for testing JH titers were collected into glass vials containing 500 μl hexane and stored at −20°C until use. The remaining carcasses of the larvae were transferred into Eppendorf tubes containing 500 μl TRIzol reagent (Invitrogen, Carlsbad, CA, USA), flash-frozen in liquid nitrogen and stored at −80°C.

RNA extraction and cDNA synthesis

After thawing and homogenization in the TRIzol reagent, RNA was extracted following the manufacturer's instructions. The quality and quantity of RNA was determined by spectrophotometry (Nanovue, GE Healthcare, Barrington, IL, USA). DNase (RNase-free, DNase kit, Applied Biosystems, Bedford. MA, USA) was added to the total RNA extract to remove trace DNA contaminants, and 1 μg of treated RNA was used for reverse transcription following an established method (Wang et al., 2009) using TaqMan reverse transcription reagents (Applied Biosystems).

Real-time quantitative PCR analyses

First-strand cDNA was used for real-time quantitative PCR (RT-qPCR) assays. Before performing the RT-qPCR, PCR amplicons from each gene were sequenced to validate the specificity of the primers (supplementary material Table S2). A dilution series of cDNA was used to establish standard curves for each gene, and amplification efficiencies were calculated based on an established method (Livak and Schmittgen, 2001; Pfaffl, 2001). After verifying that *AmILP1*, *AmILP2* and *Amrp49* primers had similar amplification efficiencies, 15 samples were randomly picked from each treatment group for expression analysis. Each biological sample was run in technical triplicate on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems) for measuring *AmILP1* and *AmILP2* transcript levels in comparison with those of the reference gene *Amrp49* by means of the ΔΔCt method (Livak and Schmittgen, 2001). Studies have shown that *Amrp49*, which has been renamed as *rpl23* (AF441189) in the honey bee genome version Amel 4.5, is stably expressed during larval development (Lourenço et al., 2008; Reim et al., 2013) and in adults (Cameron et al., 2013). Therefore *Amrp49* is commonly used as a reference gene during the larval stage (de Azevedo and Hartfelder, 2008; Martins et al., 2010) and also the adult stage of honey bees (Ben-Shahar et al., 2003; Navajas et al., 2008). RT-qPCR conditions were used as described previously for these genes (de Azevedo and Hartfelder, 2008). By monitoring negative control samples (without reverse transcriptase) and melting curve analysis, we verified that the RT-qPCR assays were not confounded by DNA contamination or primer dimers (Vandesompele et al., 2002).

Glucose and trehalose measurements

Glucose levels in the hemolymph were analyzed using a Glucose (HK) Assay Kit (Sigma, St Louis, MO, USA), following an established laboratory protocol (Hartfelder et al., 2013; Wang et al., 2012). After adding 1 ml of the glucose reagent to each hemolymph sample, these were incubated for 15 min at room temperature. A series of glucose dilutions (0, 0.5, 1, 5, 10, 30, 50 and 100 μg ml^{-1}) was prepared to set up a standard curve. After the incubation, 100 μl of each standard and sample solution was transferred in triplicate to 96-well microplates. Absorbance at 340 nm was measured using an xMark Microplate Absorbance spectrophotometer (Bio-Rad, Hercules, CA, USA) and sample glucose concentrations were calculated by linear regression. After the glucose readings were taken, 0.5 μl of trehalase (Sigma; 0.05 U ml^{-1}) was added to each well. The second reading for both standards and samples was taken after an overnight incubation at 37°C. Glucose produced from trehalose was calculated, by first subtracting the first glucose concentration value from the second total glucose concentration, then entering this into the equation: trehalose (μg)=glucose (μg)×342.3/(180.2×2).

JH radioimmunoassay

JH extraction from the 1 μl hemolymph aliquots in hexane was carried out following a liquid-phase separation protocol established for honey bee hemolymph (Huang et al., 1994). After addition of 1 ml NaCl (0.9%) and 1 ml hexane, the mixture was vigorously vortexed and the phases were separated by centrifugation (700 ***g***). The hexane phase was retrieved, and the extraction was repeated twice by adding 1 ml hexane each time. The pooled hexane phases were dried by vacuum centrifugation, and the residues were redissolved in 100 μl toluene containing 0.5% (v:v) 1,2-propanediol (Sigma) and transferred to 1.5 ml glass vials. Before starting the radioimmunoassay (RIA), the solvent was removed by vacuum centrifugation.

A JH-specific antiserum (Goodman et al., 1990), previously validated for JH detection in bees (Amdam et al., 2007; Guidugli et al., 2005), was diluted 1:1250 in phosphate buffer supplemented with BSA (0.1%) and rabbit IgG (0.1%). The assays were performed with [10-^{3}H(N)]-juvenile hormone III (specific activity

19.4 Ci mmol^{-1}, Perkin Elmer, Boston, MA, USA) diluted in phosphate buffer to 6000–6500 c.p.m. 100 µl^{-1}. Juvenile hormone III (Fluka, Buchs, Switzerland) was used as non-radioactive ligand. Standard curves were set up to cover a 50 pg to 10 ng range.

The RIA was conducted following a previously established procedure (Goodman et al., 1990) adapted for honey bees (Hartfelder et al., 2013). Samples were incubated overnight at 4°C, then supplemented with saturated ammonium sulfate (50% final concentration) to separate antibody-bound from free JH by centrifugation at 7500 ***g*** for 15 min. After washing the pellets with 50% ammonium sulfate and a novel precipitation/centrifugation step, the pellets were redissolved in 80 µl water before addition of 5 ml liquid scintillation cocktail (Optiphase Hisafe3, Perkin Elmer). Standard curve values were entered into a four-parameter fitting Excel spreadsheet specifically designed for enzyme-linked immunoassays (EIA) and RIA analyses (Bachem, Bubendorf, Switzerland; available from https://www.bachem.com/service-support/immunoassay-calculator/), based on the equation $y=\{(a–d)/[1+(x/c)^b]\}+d$, where a=maximum, b=slope, c=IC_{50} (the half-maximal inhibitory concentration) and d=minimum. Sample JH concentrations obtained by this polynomial regression were expressed as JH-III equivalents (pg µl^{-1} hemolymph).

Scoring ovariole number and additional morphological characters

Mandible and sting form, the presence of a corbicula (pollen basket) and spermatheca, and ovary size of the emerged adults were assessed under a dissecting Leica MA12 microscope (Leica, Wetzlar, Germany). Bees with more than 70 ovarioles, notched mandibles, a smooth stinger, a spermatheca and lacking a corbicula were classified as queens (Mutti et al., 2011b). Alternatively, workers were considered to have fewer than 20 ovarioles, a barbed stinger and a corbicula (Mutti et al., 2011b). Intermediates were those with 20–70 ovarioles and a mixed set of the other characters (Mutti et al., 2011b).

Statistical analysis

Gene expression data were log transformed to approximate normality (Wang et al., 2009), as verified by Bartlett and Levene's homogeneity test. A factorial ANOVA was used to test the effect of *AmILP1* and *AmILP2* dsRNAs on gene expression, followed by Fisher least significant difference (LSD) tests in *post hoc* comparisons. A Pearson correlation assay was used to reveal whether larval mass was correlated with *AmILP1* and *AmILP2* transcript levels and with JH titer. The factorial ANOVA was also used to test whether the treatment affected each morphological character such as ovariole number, mandible, stinger, corbicula or spermatheca. A principal component analysis (PCA) was applied on these multiple morphological characters to clarify general distribution patterns and separations of the bees from different treatment groups by reducing dimensions of variables. Then, a Kruskal–Wallis ANOVA was used to analyze the treatment effect on the values of sample bees given by the first principal component (PC1). These analyses were performed using STATISTICA 10.0 (StatSoft) software.

RESULTS

Quantitative validation of *AmILP1* and *AmILP2* knockdown in a full factorial experimental design

The individual whole-body RNA extracts from fifth instar larvae were assayed using an RT-qPCR protocol for *AmILP1* and *AmILP2* gene-knockdown verification (N=15). The overall effect of the factors (*AmILP1* dsRNA treatment and *AmILP2* dsRNA treatment) was determined by the main effect of a factorial ANOVA analysis. *AmILP1* transcript levels were unaffected by either *AmILP1* or *AmILP2* dsRNA treatment (factorial ANOVA, N=15, main effect of *AmILP1* dsRNA: $F_{1,56}$=0.1392, P=0.6620; main effect of *AmILP2* dsRNA: $F_{1,56}$=1.0447, P=0.3111, Fig. 1A,B). However, there was a significant decrease in *AmILP2* transcript levels in larvae with *AmILP2* dsRNA treatment (factorial ANOVA, N=15, main effect

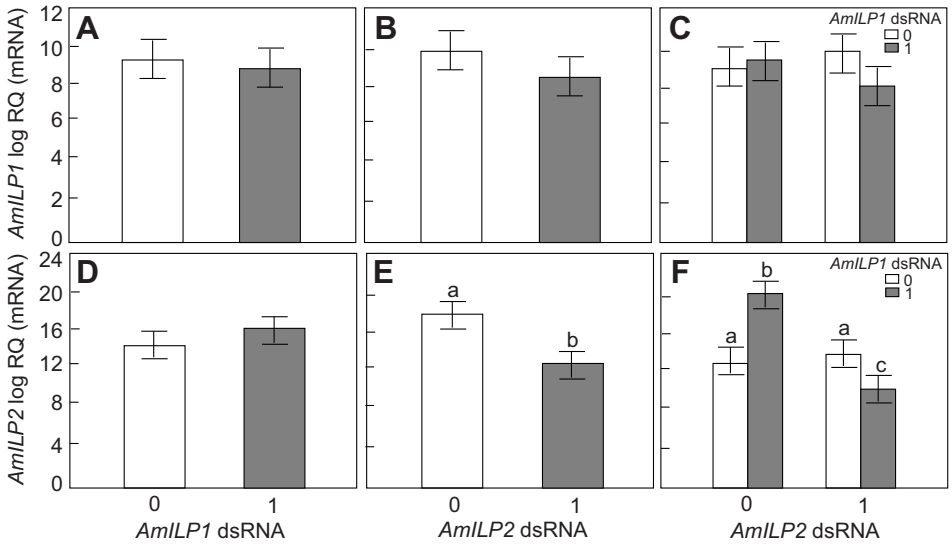

Fig. 1. *Apis mellifera* insulin-like peptide *AmILP1* and *AmILP2* gene knockdown validation in fifth instar honey bee larvae. (A,B,D,E) The main effects of *AmILP1* double-stranded (ds)RNA and *AmILP2* dsRNA on *AmILP1* and *AmILP2* gene expression in a factorial ANOVA. (C,F) The relationships between four treatment groups, as revealed by a Fisher's least significant difference (LSD) *post hoc* test: the bars from left to right represent *gfp* (green fluorescent protein), *AmILP1* dsRNA, *AmILP2* dsRNA, and *AmILP1 dsRNA* plus *AmILP2 dsRNA*. Overall, *AmILP1* gene expression was not affected by *AmILP1* dsRNA (A) and *AmILP2* dsRNA (B). Overall, *AmILP2* gene expression was not affected by *AmILP1* dsRNA (D), but was significantly reduced by *AmILP2* dsRNA (E). Panel F shows that compared with *gfp* (first bar): (i) *AmILP1* dsRNA treatment increased *AmILP2* expression level; (ii) *AmILP2* dsRNA in the absence of *AmILP1* dsRNA did not reduce the *AmILP2* mRNA level; (iii) but when combined with *AmILP1* dsRNA application, *AmILP2* was significantly downregulated. Data are presented as means ± s.e.m. (N=15). RQ is relative quantification. Different letters (a–c) over the bars indicate significant differences between treatments. '0' represents no dsRNA treatment and '1' represents dsRNA treatment.

of *AmILP2* dsRNA: $F_{1,56}$=20.9941, P<0.0001, Fig. 1E), but not in those treated with *AmILP1* dsRNA (factorial ANOVA, N=15, main effect of *AmILP1* dsRNA; $F_{1,56}$=1.5382, P=0.2201, Fig. 1D). As the main effect of *AmILP2* dsRNA on *AmILP2* gene expression shown in Fig. 1E includes the effect of *AmILP2* dsRNA at two levels of *AmILP1* dsRNA treatment (0 and 1), this result indicates that *AmILP2* dsRNA, independent or not independent of *AmILP1* dsRNA, significantly downregulated the expression of its target gene at the whole-body level.

In order to determine whether *AmILP1* dsRNA contributed to the main effect of *AmILP2* dsRNA on *AmILP2* expression, we looked at the interactions between *AmILP1* dsRNA and *AmILP2* dsRNA treatment. We found that there was no interaction between these two treatments on *AmILP1* expression (factorial ANOVA, interaction effect: $F_{1,56}$=2.1251, P=0.1505, Fig. 1C) but there was a significant interaction on *AmILP2* gene expression (factorial ANOVA, $F_{1,56}$=41.5698, interaction effect: P<0.0001, Fig. 1F), suggesting that the reduction of *AmILP2* expression by *AmILP2* dsRNA (Fig. 1E) was dependent on the level of *AmILP1* dsRNA treatment. Next, we performed a Fisher's LSD *post hoc* test to further dissect how the four treatment groups (*gfp*, *AmILP1* dsRNA, *AmILP2* dsRNA, and *AmILp1* dsRNA plus *AmILP2* dsRNA) contributed to the main effects of *AmILP2* dsRNA in this study. We found that, compared with *gfp* controls, (i) single *AmILP1* dsRNA treatment actually increased *AmILP2* transcript level (P<0.0001, Fig. 1F), (ii) single *AmILP2* dsRNA treatment, on its own, did not significantly reduce *AmILP2* mRNA levels (P=0.1985), but (iii) the combined *AmILP1* dsRNA and *AmILP2* dsRNA significantly decreased *AmILP2* transcript abundance (P=0.0405). These results suggest that the level of *AmILP1* dsRNA treatment contributed to the significant main effect of *AmILP2* dsRNA in a whole larva (Fig. 1E): the application of *AmILP2* dsRNA alone did not cause a reduction in the *AmILP2* mRNA level, but *AmILP1* dsRNA application enhanced the effect of *AmILP2* dsRNA – resulting in a significant decrease in *AmILP2* transcript abundance in Fig. 1E.

Glucose and trehalose titers in the hemolymph

Studies in *Drosophila* indicated that *ILPs* in the brain were involved in regulating carbohydrate metabolism and blood sugar titers (Broughton et al., 2005; Saltiel and Kahn, 2001), and we previously found that *AmILP1* gene expression in adult honey bees was linked with blood sugar levels (Wang et al., 2012). Here, we measured carbohydrate reserves (glucose and trehalose) in the hemolymph in order to test whether the *AmILPs* may directly regulate blood sugar titers during honey bee larval development. We found that neither glucose nor trehalose concentration was influenced by either *AmILP1* dsRNA or *AmILP2* dsRNA treatment (factorial ANOVA, main effect of *AmILP1* dsRNA: N=20, $F_{1,76,\text{glucose}}$=0.1310, P=0.7184 and $F_{1,76,\text{trehalose}}$=0.11530, P=0.6968; main effect of *AmILP2* dsRNA: $F_{1,76,\text{glucose}}$=0.8100, P=0.1825 and $F_{1,76,\text{trehalose}}$=0.7659, P=0.3843, Fig. 2A,B,D,E). There was also no interaction between *AmILP1* dsRNA and *AmILP2* dsRNA treatment on the content of either sugar (factorial ANOVA, $F_{1,76,\text{glucose}}$=0.9799, P=0.3254; $F_{1,76,\text{trehalose}}$=0.1190, P=0.7311, Fig. 2C,F). Considering that only *AmILP2* knockdown was validated statistically at the whole-body RNA level in larvae, we infer that *AmILP2* does not directly regulate hemolymph carbohydrate reserves in honey bee larvae.

Hemolymph JH titers in *AmILP1* and *AmILP2* dsRNA-treated larvae

JH is a central regulator controlling queen caste development, and its levels can be regulated by EGF signaling (Kamakura, 2011) and affected by both *IRS* and *TOR* knockdown (Jin and Esteva, 2008; Mutti et al., 2011a). Therefore, examining whether JH is affected by *AmILP* knockdown is key to understanding the relationship between JH and IIS in honey bee larvae. By measuring the JH titers in larval hemolymph by means of a specific RIA, we found that the JH titers were significantly decreased by *AmILP1* dsRNA (factorial ANOVA, N=16–19, $F_{1,67}$=5.0970, P=0.0272, Fig. 3A), but not by *AmILP2* RNAi (factorial ANOVA, N=16–19, $F_{1,67}$=1.7474, P=0.1907, Fig. 3B). There was no significant interaction effect between *AmILP1* and *AmILP2* dsRNA treatments (factorial ANOVA, $F_{1,76}$=1.4309, P=0.2358, Fig. 3C), indicating that the effect of *AmILP1* dsRNA on JH was independent of *AmILP2* dsRNA. *Post hoc* analysis further showed that the larvae treated with *AmILP1* dsRNA (Fisher LSD: P=0.0114) and the larvae treated with combined *AmILP1* and *AmILP2* dsRNA (Fisher LSD: P=0.0135) had lower JH levels compared with *gfp* controls (Fig. 3C). As we could not verify a knockdown in terms of *AmILP1* transcript level in the same individual larva, these results raised an interesting question about whether the change in JH titers in the bees treated with *AmILP1* dsRNA was specific. It is, however, plausible that the whole-body RNA levels measured may have masked changes in

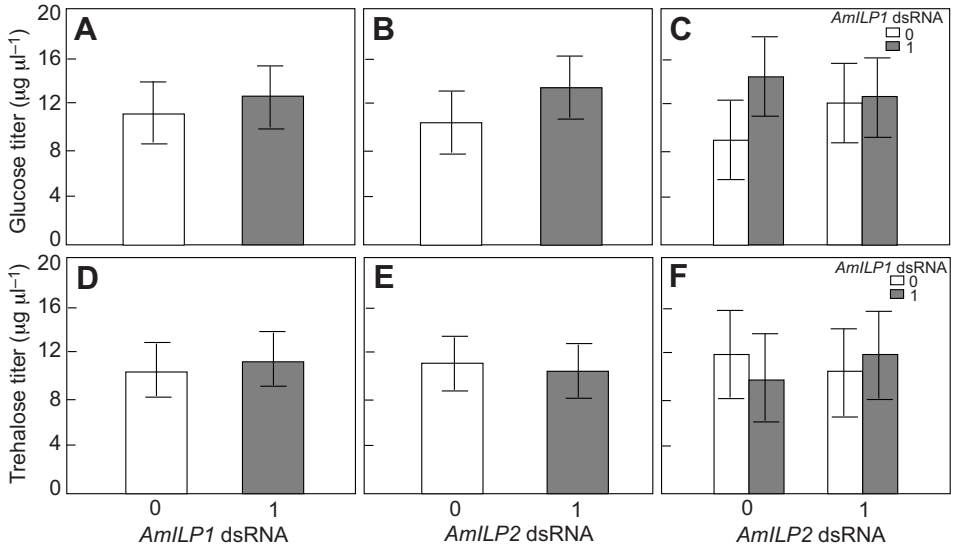

Fig. 2. Glucose and trehalose levels in the hemolymph of larvae treated with *AmILP1* and *AmILP2* dsRNA. (A,B,D,E) The main effects of *AmILP1* dsRNA and *AmILP2* dsRNA on glucose and trehalose levels in a factorial ANOVA. (C,F) The relationships between four treatment groups by LSD *post hoc* test: the bars from left to right represent *gfp*, *AmILP1* dsRNA, *AmILP2* dsRNA, and *AmILP1* dsRNA plus *AmILP2* dsRNA. There was no main effect of either *AmILP1* dsRNA or *AmILP2* dsRNA on glucose (A,B) and trehalose (D,E) titers. There was no difference in the four treatment groups with respect to glucose and trehalose titers (C,F). Data are shown as means ± s.e.m. (N=20). '0' represents no dsRNA treatment and '1' represents dsRNA treatment.

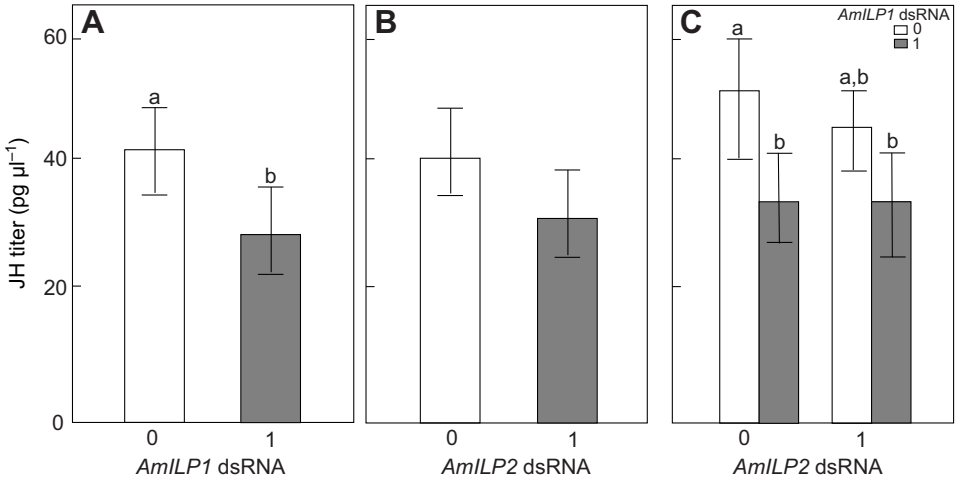

Fig. 3. Hemolymph juvenile hormone (JH) titer in larvae treated with *AmILP1* and *AmILP2* dsRNA. (A,B,D,E) The main effects of *AmILP1* dsRNA and *AmILP2* dsRNA on JH titer in a factorial ANOVA. (C,F) The relationships between four treatment groups as revelaed by a LSD *post hoc* test: the bars from left to right represent *gfp*, *AmILP1* dsRNA, *AmILP2* dsRNA, and *AmILP1* dsRNA plus *AmILP2* dsRNA. There was a significant main effect of *AmILP1* dsRNA on JH titer (A), but no main effect of *AmILP2* dsRNA on the JH titer (B). Panel C shows that, compared with *gfp*, both single *AmILP1* dsRNA treatment and *AmILP1* dsRNA plus *AmILp2* dsRNA treatment reduced JH titer, but single *AmILP2* dsRNA treatment did not do so, indicating that only *AmILP1* dsRNA contributed to the reduction of JH titer in A. Data are presented as means ± s.e.m. (*N*=16–19). Different letters indicate significant differences among treatments. '0' represents no dsRNA treatment and '1' represents dsRNA treatment.

AmILP1 transcript levels that only occurred in a small subset of cells, such as in the neuroendocrine axis.

Body mass

There is a correlation between body mass and ovariole number in honey bees (Linksvayer et al., 2011; Snodgrass, 1956), but a recent *in vitro* rearing study has shown that body size can be independent of ovariole number and other queen phenotype characters (Linksvayer et al., 2011), indicating that caste morphological traits may be regulated by separate pathways (Kamakura, 2011). In our study, the body mass of fifth instar larvae was not affected by either *AmILP1* dsRNA or *AmILP2* dsRNA (factorial ANOVA, *N*=20, main effect of *AmILP1* dsRNA: $F_{1,76}$=1.3834, *P*=0.2432; main effect of *AmILP2* dsRNA, $F_{1,76}$=1.6561, *P*=0.2020, Fig. 4A,B), but there was a significant interaction effect between *AmILP1* and *AmILP2* dsRNAs (factorial ANOVA, $F_{1,76}$=5.5990, *P*=0.0205, Fig. 4C). These results suggest that the effect of *AmILP1* dsRNA on the body mass of fifth instar larvae depends on the level of *AmILP2* dsRNA: *AmILP1* dsRNA increased the body mass in the absence of *AmILP2* dsRNA, but combined *AmILP1* dsRNA and *AmILP2* dsRNA treatment tended to reduce body mass. *Post hoc* analysis further showed that larvae treated with *AmILP1* dsRNA were heavier than *gfp* control larvae (*post hoc* LSD: *P*=0.0010) and larvae treated with both *AmILP1* and *AmILP2* dsRNAs (*post hoc* LSD: *P*=0.0465).

As AmILPs and JH are thought to be interconnected in the regulation of honey bee caste development, we also plotted the respective larval mass against *AmILP1* and *AmILP2* expression and JH titers to explore putative associations. For both *AmILP1* and *AmILP2*, we found significant positive correlations with larval mass (Pearson correlation, *N*=57, *AmILP1*: *P*=0.0250; *AmILP2*: *P*=0.0092, Fig. 5A,B), suggesting that both *AmILP* genes are involved in regulating larval development in either a direct or an indirect way, supporting a hypothesis for general functions of *AmILP1* and *AmILP2* in honey bee development. In contrast, JH

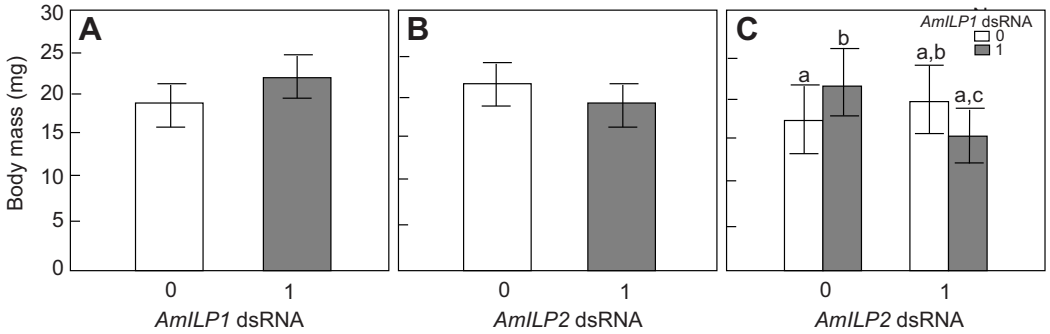

Fig. 4. Effects of *AmILP1* and *AmILP2* dsRNA treatment on body mass of fifth instar larvae. (A,B,D,E) The main effects of *AmILP1* dsRNA and *AmILP2* dsRNA on body mass in a factorial ANOVA. (C,F) The relationships between four treatment groups as revealed by a LSD *post hoc* test: the bars from left to right represent *gfp, AmILP1* dsRNA, *AmILP2* dsRNA, and *AmILP1* dsRNA plus *AmILP2* dsRNA. There was no main effect of either *AmILP1* dsRNA or *AmILP2* dsRNA on body mass (A,B). Panel C shows that *AmILP1* dsRNA treatment increased body mass in the absence of *AmILP2* dsRNA, but the combination of *AmILP1* dsRNA and *AmILP2* dsRNA tended to reduce body mass. Together with the result of a significant interaction between *AmILP1* dsRNA and *AmILP2* dsRNA treatments on body mass, these results suggest that the effect of *AmILP1* dsRNA on body mass of the fifth instar larvae depends on the level of *AmILP2* dsRNA. Data are represented as means ± s.e.m. (*N*=20). Different letters indicate significant differences among treatments. '0' represents no dsRNA treatment and '1' represents dsRNA treatment.

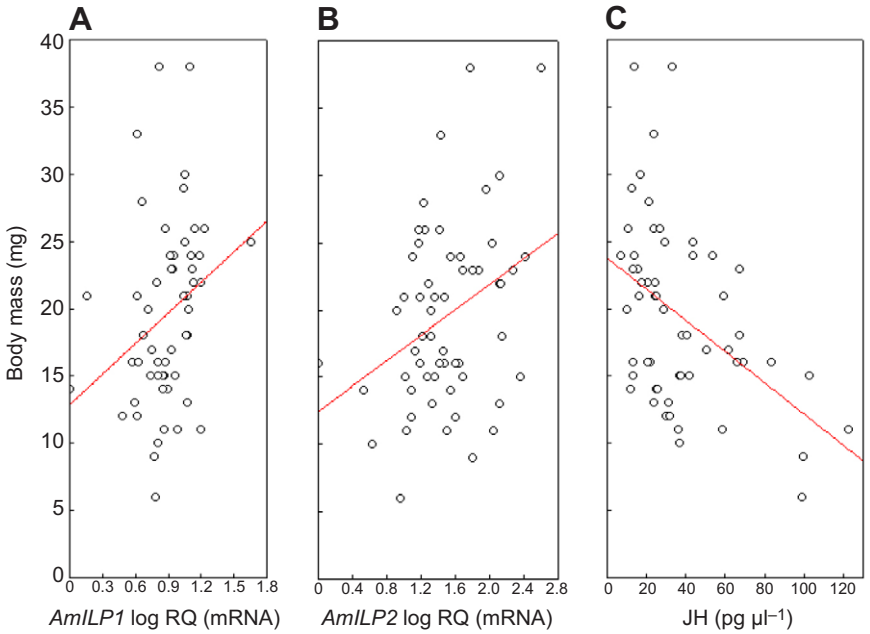

Fig. 5. Correlation between larval body mass and *AmILP* expression or JH level. *AmILP1* transcript abundance was positively correlated with body mass (A); *AmILP2* transcript abundance was also positively correlated with body mass (B); but JH titer was negatively correlated with body mass (C). RQ is relative quantification. Circles represent individual larvae from all four RNA interference (RNAi) treatments; regression lines were obtained by Pearson correlation analysis.

titers were negatively correlated with larval mass (Pearson correlation, N=57, P=0.0004, Fig. 5C). This negative correlation seems contradictory to the general role of JH in honey bee caste differentiation. However, the fifth instar is a critical stage to initiate honey bee metamorphosis, coordinated in concert by JH and ecdysteroid titers. Perhaps such dynamic changes (temporal or rapid) resulted in the negative relationship between JH and body mass. Nonetheless, further investigation is needed to test this hypothesis.

Ovariole number and other morphological traits

Ovaries were dissected and ovariole number was counted after adult eclosion. We found that ovariole number was significantly reduced in bees subject to *AmILP2* RNAi (factorial ANOVA, N=12–27, $F_{1,67}$=5.2069, P=0.0257, Fig. 6B) but not to *AmILP1* dsRNA (factorial ANOVA, N=12–27, $F_{1,67}$=1.1859, P=0.2801, Fig. 6A). There was no interaction between *AmILP1* and *AmILP2* dsRNA treatments (factorial ANOVA, $F_{1,67}$=0.0642, P=0.8008, Fig. 6C), suggesting *AmILP2* dsRNA reduced ovariole number independent of *AmILP1* dsRNA. *Post hoc* analysis showed that bees treated with *AmILP2* dsRNA (Fisher LSD: P=0.0067) and the bees treated with a combination of *AmILP1* and *AmILP2* dsRNA (Fisher LSD: P=0.0570) had significantly fewer ovarioles than bees treated with *AmILP1* dsRNA alone.

Other morphological characters, such as mandible shape, stinger shape, size of spermatheca and presence/absence of a corbicula, were also monitored based on an established protocol (Mutti et al., 2011b; Patel et al., 2007). There was no main effect of either *AmILP1* dsRNA or *AmILP2* dsRNA, and no interaction effect on any of these morphological characters (factorial ANOVA, P>0.05; detailed results can be found in supplementary material Table S3).

A PCA was utilized to clarify general patterns, similarities or separations of cases between the different treatment groups by reducing the dimensions of morphological variables. This revealed that 73.25% of the total variation can be explained by the first principal component (PC1), and 9.61% of the remaining total variation can be explained by the second principal component (PC2). The eigenvalue of PC1 was 3.6623, and other PCs did not exceed 1 (supplementary material Table S4), meaning that PC1 contributed

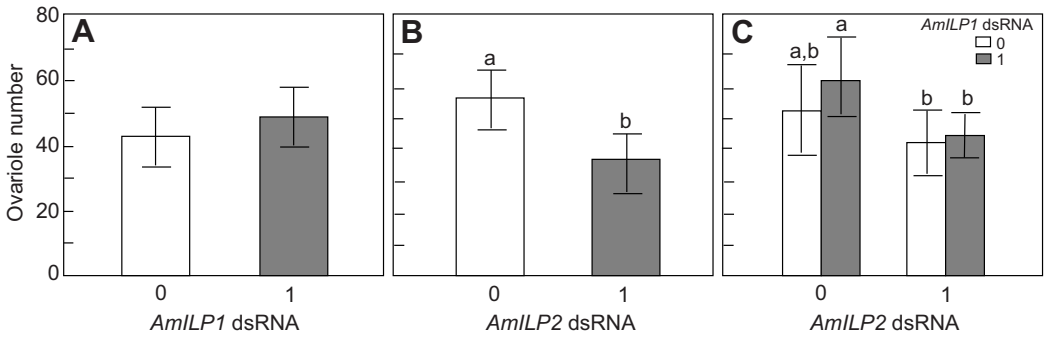

Fig. 6. Effects of *AmILP1* and *AmILP2* dsRNA treatment on ovariole number of newly emerged adults. (A,B) The main effects of *AmILP1* dsRNA and *AmILP2* dsRNA on ovariole number in a factorial ANOVA. (C) The relationships between the four treatment groups by LSD *post hoc* test: the bars from left to right represent *gfp*, *AmILP1* dsRNA, *AmILP2* dsRNA, and *AmILP1* dsRNA plus *AmILP2* dsRNA. Even though there was no main effect of *AmILP1* dsRNA on ovariole number (A), ovariole number was significantly reduced by *AmILP2* RNAi (B). Panel C shows that compared with *gfp*, both *AmILP2* dsRNA treatment alone and *AmILP1* dsRNA plus *AmILP2* dsRNA treatment tended to reduce ovariole number, whereas *AmILP1* dsRNA treatment tended to increase ovariole number. As we did not find a significant interaction between *AmILP1* and *AmILP2* dsRNA treatment on ovariole number (C), these results suggest that the main effect of *AmILP2* dsRNA on ovary development in B is independent of *AmILP1* dsRNA. Data are presented as means ± s.e.m. (N=12–27). Different letters indicate significant differences among treatments. '0' represents no dsRNA treatment and '1' represents dsRNA treatment.

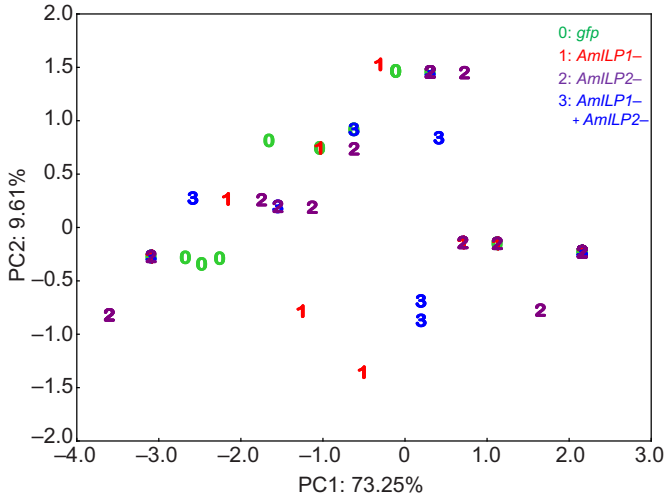

Fig. 7. Score plot of PC1 and PC2 of a principal component analysis (PCA) on the multiple morphological traits. There is no clear separation of the bees among the treatment groups.

more to the variance than the original variables, but other PCs could be considered as sampling noise. All the variables (morphological characters) contributed almost equally to PC1 (contributions: 17–22%, supplementary material Table S5). Analysis of PC1 *versus* PC2 (Fig. 7) revealed that along PC1 there was no clear separation among cases (bees) according to treatment. Next, we carried out a Kruskal–Wallis ANOVA to test whether the distribution of samples in treatment groups in PC1 differed. There was no difference in the distribution of samples among the treatment groups in PC1 (the combined variable) (Kruskal–Wallis ANOVA: χ^2=4.7526, P=0.1908), suggesting that the treatments did not significantly influence queen–worker caste differentiation, which is characterized by these multiple morphological characters, though *AmILP2* RNAi significantly affected ovariole number.

DISCUSSION

By using a gene knockdown approach, we herein performed the first functional study to investigate the role of insulin peptide-encoding genes, *AmILP1* and *AmILP2*, in queen–worker differentiation during honey bee larval development. Our data show that *AmILP2* expression was susceptible to *AmILP2* RNAi when *AmILP1* dsRNA was used simultaneously, which resulted in diminished transcript levels in the whole larval body. Although *AmILP2* dsRNA did not cause any change in hemolymph JH levels, it had an effect on ovariole number of adult bees. In contrast, *AmILP1* expression at the whole-body level was not affected by *AmILP1* dsRNA treatment, but the hemolymph JH levels in these larvae were significantly reduced by the treatment. Thus, a general conclusion that can be drawn from these results is that *AmILP1* and *AmILP2* dsRNAs have differential efficacies to downregulate the target genes in the whole larval body.

Efficacies of *AmILP1* and *AmILP2* RNAi in the fat body

RNAi efficacy is affected by many factors, such as the specificity of the dsRNA, the RNAi delivery method, the expression level of the gene, cell types in the target tissue and the nature of the regulatory machinery of RNAi. In honey bees, both AmILP-encoding genes are represented by a single copy each in the honey bee genome (de Azevedo and Hartfelder, 2008). Their transcripts are relatively short (around 400 bp), and there is no evidence for transcript variants. Additionally, the dsRNAs were designed to target 260–280 bp regions of *AmILP1* and *AmILP2*, and no off-target matches were found by alignments against the honey bee genome. Furthermore, the final concentration of dsRNA (200 μg ml^{-1}) in the larval diet and the *in vitro* rearing protocol has been validated in previous *TOR* and *IRS* knockdown studies (Mutti et al., 2011b; Patel et al., 2007). In this study, we achieved an overall 30% reduction in *AmILP2* transcript levels by *AmILP2* RNAi. Therefore, it is unlikely that dsRNA specificity and the protocol have issues resulting in the differential efficacies between *AmILP1* and *AmILP2* RNAi.

A factor that may explain why we did not achieve a significant *AmILP1* gene knockdown could be the low level of *AmILP1* expression in early fifth instar larvae (de Azevedo and Hartfelder, 2008). So, the difficulty encountered in achieving *AmILP1* knockdown could be related to the general difficulty in downregulating a gene with low transcript abundance. Additionally, differential cell-type specificities between *AmILP* gene expression and dsRNA targeting may be another reason for the differential RNAi efficacies. In adult honey bees, the tissue that best responds to dsRNA treatment is the fat body (Amdam et al., 2003; Jarosch and Moritz, 2011), and it is also the predominant tissue type in larvae. The insect fat body is composed of two cell types, trophocytes and oenocytes. A recent study has revealed that the expression of *AmILP1* and *AmILP2* in honey bee fat body has different cell specificities: *AmILP1* is highly expressed in oenocytes and *AmILP2* is expressed strongly in both oenocytes and trophocytes (Nilsen et al., 2011). However, the preferential uptake characteristics of dsRNA molecules by oenocytes and trophocytes are different, with trophocytes uptaking considerably more dsRNA than oenocytes (Jarosch and Moritz, 2011). Therefore, the lack of a significant *AmILP1* knockdown in our experiments may be due to the poor *AmILP1* dsRNA uptake capability of oenocytes (Jarosch and Moritz, 2011), as well as the low transcript abundance of *AmILP1* in the developmental stage. As gene knockdown is dose dependent, increasing the dosage or extending dsRNA feeding time may raise the success rate for knocking down the *AmILP1* gene in the fat body in future studies.

In this study, the *AmILP2* gene was not directly knocked down when its dsRNA was applied alone. One of the reasons could again be a low level of *AmILP2* expression in fifth instar larvae (de Azevedo and Hartfelder, 2008). However, we found that *AmILP2* dsRNA significantly downregulated *AmILP2* gene expression when *AmILP1* dsRNA was applied simultaneously, whereas *AmILP1* dsRNA treatment caused an increase in *AmILP2* mRNA. Though there is no simple explanation for this phenomenon, it is worthy of note that the effect of RNAi can be physiologically amplified and systemically spread in some organisms including *Caenorhabditis elegans* and certain insects (Tomoyasu et al., 2008; Miller et al., 2012). These processes involve RNA-directed RNA polymerase activity, which depends on high levels of expression of target RNA (Dougherty and Parks, 1995; Sijen et al., 2001). Therefore, the potency of *AmILP2* dsRNA might have been enhanced once the *AmILP2* transcript level was increased by *AmILP1* dsRNA. As the RNAi machinery includes both transcriptional and post-transcriptional gene silencing modes (Noma et al., 2004), this can involve complex negative and positive feedback (Xie et al., 2003; Grewal and Elgin, 2007). In addition, regulatory mechanisms in RNAi vary among organisms (Tomoyasu et al., 2008), and the way in which RNAi is controlled and regulated in insects is still poorly understood. Therefore, future studies directed towards detecting and identifying regulatory mechanisms of RNAi in insects are likely to

shed light on the question of how *AmILP1* dsRNA could enhance the effect of *AmILP2* dsRNA.

Potential relationship of brain AmILP1 to JH production and AmILP2

Interestingly, we observed a significant reduction of JH in response to *AmILP1* dsRNA treatment, even though no significant downregulation was achieved for this gene at the whole-body level. It is worthy of note that the majority of ILPs are produced in the brain of most insect species (Antonova et al., 2012; Brogiolo et al., 2001; Iga and Smagghe, 2011; Riehle et al., 2006), including honey bees (Ament et al., 2008; Corona et al., 2007) and *Drosophila* (Brogiolo et al., 2001). And JH is synthesized in the closely associated CA of the insect retrocerebral complex (Goodman and Cusson, 2012), as also shown for honey bee larvae (Rachinsky and Hartfelder, 1990). In *Drosophila*, the small cluster of AmILP-producing neuroendocrine cells was shown to transmit ILPs to the JH-producing CA (Krieger et al., 2004) by axons directly projecting to the ring gland (Cao and Brown, 2001; Géminard et al., 2006). In line with these findings (Lane and Swales, 1978; Restifo et al., 1995), we hypothesize that a cluster of AmILP-producing cells in the brain of honey bee larvae may have been targeted by *AmILP1* dsRNA, which consequently affected JH production in the CA. Although the adult honey bee brain has been shown to be resilient to dsRNA treatment (Farooqui et al., 2004; Jarosch and Moritz, 2011), it is possible that the larval hemolymph–brain barrier could be more leaky than that of adults (Lane and Swales, 1978; Restifo et al., 1995), especially during the onset of metamorphosis.

Furthermore, several studies have provided evidence for a positive regulation of *ILP* expression by JH in many insect species including *Drosophila* (Corona et al., 2007; Sheng et al., 2011; Tu et al., 2005). Reciprocally, it was found that JH synthesis was modulated by brain *Drosophila* ILPs (Tatar et al., 2003). In honey bees, treatment with methoprene (a JH analog) positively affected brain *AmILP1* levels in both adult queens and workers (Corona et al., 2007). Our recent study also suggested that *AmILP1* expression in the fat body is negatively linked to hemolymph JH titers in adult worker bees (Wang et al., 2012). In addition, a connection between *AmILP1* expression and JH synthesis was proposed based on the temporal coincidence between the peaks of *AmILP1* expression and JH titers in honey bee larvae (Wheeler et al., 2006). Finally, interference with downstream regulators of IIS and/or EGF signaling, such as the *IRS* (Mutti et al., 2011b) and *TOR* genes (Patel et al., 2007), resulted in a decrease in JH titers (Mutti et al., 2011a). Taken together, the reduction in JH levels seen as a result of *AmILP1* dsRNA treatment is likely a specific effect of *AmILP1* RNAi, and our study provides the first evidence that brain *AmILP1* may regulate JH production in honey bee larvae.

Finally, our study indicates that the expression of brain *AmILP1* and fat body *AmILP2* is correlated in honey bees. In *Drosophila*, overexpression of insulin-like peptides (DILPs) in the fat body inhibited brain DILP secretions (Bai et al., 2012), and gene knockouts of *DILPs* in the brain caused synergy and compensation of expression of *DILPs* in the fat body (Grönke et al., 2010). In honey bees, previous studies have suggested that AmILP1 and AmILP2 act as an agonist and an antagonist, respectively, of InRs in the brain regulating JH secretion (Nilsen et al., 2011). However, how brain AmILPs connect with fat body AmILPs is poorly understood. Here, we found that *AmILP1* dsRNA was able to increase *AmILP2* transcript abundance in the whole body of fifth instar larvae (Fig. 1F), with the fat body making the major tissue contribution. As *AmILP1* dsRNA probably affects brain *AmILP1* secretion in fifth instar larvae, our findings suggest that fat body *AmILP2* compensates for the downregulation of brain *AmILP1*, thus representing a circuitry similar to that found in *Drosophila*. Although *AmILP1* dsRNA also induced a decrease in JH titers, we did not find any correlation between JH and fat body *AmILP2* levels in these fifth instar larvae (supplementary material Fig. S1), suggesting that JH is not involved in this hypothetical compensatory response of fat body *AmILP2*.

Roles of JH and *AmILP2* in worker caste development

Experimental and modeling evidence supports the suggestion that an elevated JH titer during the fourth and early fifth instar of honey bees inhibits the induction of autophagic cell death in the larval ovary (Schmidt Capella and Hartfelder, 2002), and rescues the queen phenotype after *IRS* and/or *TOR* gene knockdown (Mutti et al., 2011a). Therefore, a logical conclusion would be that decreasing JH levels would promote ovary degradation and induce the worker phenotype. In our study, however, the main effect of *AmILP1* dsRNA was an ~40% reduction in JH titers, but there was no apparent effect on ovary degradation and caste characters in general, suggesting that other regulators in addition to a low JH titer may be required for full worker phenotype development. This is supported by the finding that the downregulation of *AmILP2* transcript abundance in the fat body, in addition to an ~35% (though statistically not significant) reduction in JH titers in these larvae was associated with fewer ovarioles. Together with the fact that downregulation of *IRS* and *TOR* in the larval fat body reduces JH, this suggests that the fat body secretes regulators that modulate CA activity.

Moreover, our results indicate that *AmILP1* and *AmILP2* have different roles during honey bee larval development, which is also consistent with their expression profiles (de Azevedo and Hartfelder, 2008). In *Drosophila*, different ILPs show tissue-specific functions. Whereas brain ILPs tend to regulate energy metabolism and control hemolymph sugar titers (Broughton et al., 2005; Rulifson et al., 2002), fat body ILPs are the functional equivalent of insulin-like growth factor 1 (IGF1), modulating cell proliferation and organ growth (Okamoto et al., 2009). It has already been suggested that AmILP2 may act as an IGF in the larval fat body of honey bees (Wheeler et al., 2006), but functional evidence for this hypothesis was lacking. Our data now indicate that *AmILP2* knockdown in the fat body does not modulate hemolymph sugar levels during larval development, but instead *AmILP2* is more related to ovary development and body mass in larvae.

Our study suggests that fat body *AmILP2* more likely contributes to regulating ovariole development rather than all worker traits, as *AmILP2* knockdown did not significantly affect the expression of other worker traits. Additionally, both JH and ILPs are involved in anti-apoptosis in many other insect species (Schmidt Capella and Hartfelder, 2002; Johnson et al., 2006). Therefore, *AmILP2* knockdown in the fat body may mediate ovary degradation at the end of larval development. As *AmILP2* knockdown did not change JH titers and the *AmILP2* mRNA level was not significantly correlated with JH titers, *AmILP2* may be indirectly connected with JH through other regulators such as *AmILP1*. Clearly, further studies are needed to test the hypothesis.

Moreover, ovary size and body size in adult bees generally are correlated (Linksvayer et al., 2011), suggesting their regulatory pathways may have common elements. Studies in *Drosophila* showed that fat body *DILPs* are involved in regulating body size (Okamoto et al., 2009) and fat cell mass (DiAngelo and Birnbaum, 2009). Here, we found that *AmILP1* dsRNA treatment significantly increased *AmILP2* transcript abundance at the whole-body level

(mainly fat bodies), and also increased the body mass of fifth instar larvae, leading us to infer that fat body *AmILP2* in honey bee larva may also play a role in determining body mass.

CONCLUSIONS

To summarize, by means of an RNAi approach we demonstrated that *AmILP2* expressed in the fat body is directly involved in the expression of a queen-type ovary during honey bee caste development, whereas *AmILP1* may have an indirect effect *via* modulation of JH production in the CA. Thus, we propose that the regulation of worker caste development is not simply a reversed pathway of queen caste development; instead, a network of regulators must cooperate with JH to drive worker development. Furthermore, rather than being an insulin-like peptide, the function of AmILP2 appears to be similar to IGF, regulating cell and organ growth. In agreement with other studies (Kamakura, 2011; Mutti et al., 2011a), our study supports the suggestion that the IIS pathway has a modulatory and probably only minor role in caste development of honey bees. Nonetheless, as binding affinities of AmILPs to AmInRs have not yet been investigated in honey bees, the exact role of AmILPs and AmInRs, especially their interactions with other local signaling and endocrine pathways, are still puzzling in our understanding of honey bee development and physiology.

LIST OF ABBREVIATIONS

AmILP	*Apis mellifera* insulin-like peptide
AmInR	*Apis mellifera* insulin receptor
CA	corpora allata
DILP	*Drosophila* insulin-like peptide
dsRNA	double-stranded RNA
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GFP	green fluorescent protein
IGF	insulin-like growth factor
IIS	insulin/insulin-like growth factor 1 signaling
ILP	insulin-like peptide
IRS	insulin receptor substrate
JH	juvenile hormone
RNAi	RNA interference
RT-qPCR	real-time quantitative PCR
TOR	target of rapamycin

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AUTHOR CONTRIBUTIONS

Y.W., G.V.A. and S.V.A. conceived and designed the experiment. Y.W., S.V.A. and K.H. conducted the experiment. Y.W. analyzed the data. K.H. and G.V.A contributed reagents and materials. Y.W., K.H. and G.V.A. wrote the manuscript.

COMPETING INTERESTS

No competing interests declared.

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