

RESEARCH ARTICLE

Ovary ecdysteroidogenic hormone activates egg maturation in the mosquito *Georgecraigius atropalpus* after adult eclosion or a blood meal

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SUMMARY

The rockpool mosquito, *Georgecraigius atropalpus*, is a facultatively autogenous species that produces its first egg clutch without a blood meal shortly after emergence. Several days after depositing this clutch, females must take a blood meal to produce a second egg clutch. Decapitation of females shortly after emergence or blood ingestion prevents egg maturation. Here, we report that a single injected dose of the neuropeptide ovary ecdysteroidogenic hormone (OEH) fully restored egg maturation in decapitated females in both circumstances. This neuropeptide and two insulin-like peptides (ILPs) are potent gonadotropins in the related yellow fever mosquito, *Aedes aegypti*. ILP3 was marginally restorative in decapitated *G. atropalpus*, and ILP4 had no effect. Egg maturation in non- and blood-fed *G. atropalpus* was dependent on the enzymatic mobilization of amino acids from stored protein or the blood meal for yolk protein (vitellogenin, VG) synthesis and uptake by oocytes. We further show that OEH stimulates serine protease activity in the fat body of newly eclosed females or in the midgut of blood-fed ones, and ecdysteroid hormone production by the ovaries of both females. In contrast, only 20-hydroxyecdysone stimulated VG synthesis in the fat body of non- and blood-fed females. Using RNA interference to knock down expression of the insulin receptor, we found that OEH still fully restored autogenous egg maturation. In summary, our results identify OEH as a primary regulator of egg maturation in both autogenous and blood-fed *G. atropalpus* females and suggest the shift from blood meal-dependent to blood meal-independent release of OEH is a key factor in the evolution of autogeny in this species.

Key words: neuropeptide, insulin-like peptides, ecdysteroid hormone, autogeny, anautogeny.

Received 3 May 2012; Accepted 11 July 2012

INTRODUCTION

Mosquitoes (Diptera: Culicidae) form a monophyletic assemblage that is currently divided into two subfamilies (Anophelinae and Culicinae), 44 genera and >3500 species (Harbach and Kitching, 1998; Harbach, 2007; Bertone et al., 2008; Reidenbach et al., 2009). Typically, adult mosquitoes obtain nutrients for survival and reproduction from three sources (Clements, 1992; Foster, 1995): (1) teneral reserves from larval feeding on the microbiota and detritus in water, (2) nectar or other plant juices for energy, and (3) blood taken only by females for egg production. Mosquito species for which females require a blood meal to mature eggs are called anautogenous (Roubaud, 1929). Virtually all anautogenous species lay multiple clutches of eggs, and development of each clutch depends upon the female consuming one or more blood meals (Clements, 1992; Briegel, 2003). Some mosquito species are referred to as facultatively autogenous because the females mature a first clutch of eggs without a blood meal, but then must blood feed to produce subsequent clutches of eggs (Rioux et al., 1975; O'Meara and Lounibos, 1981; O'Meara, 1985; Attardo et al., 2005; Provost-Javier et al., 2010). A few species are obligately autogenous because females produce eggs without ever blood feeding (O'Meara et al., 1981; Masler et al., 1983).

Phylogenetic studies indicate an ancient origin for the Culicidae with divergence of the lineages leading to the Anophelinae and Culicinae occurring approximately 217 million years ago (Bertone et al., 2008; Reidenbach et al., 2009). The basal position of the Anophelinae together with nearly all known anophelines being

anautogenous also strongly suggests that the common ancestor of mosquitoes had to blood feed to produce eggs. However, prior surveys (Rioux et al., 1975) as well as our more recent search of the literature indicates that facultative or obligate autogeny has arisen multiple times in the Culicinae. A key question in mosquito life history, therefore, is how do anautogenous species regulate egg formation and what changes in this regulatory program or its initiation have led to the evolution of autogeny?

Egg development among anautogenous species is best understood in the yellow fever mosquito, *Aedes aegypti* (Culicinae, Tribe Aedini), which transmits several pathogens that cause disease in humans including Dengue fever and Yellow fever. Upon eclosion, adult females enter a pre-vitellogenic phase where the corpora allata secrete juvenile hormone (specifically JH III) (Li et al., 2003). This programs reproductive competency by promoting the expression of the target of rapamycin (TOR) and ecdysteroid hormone (ECD) signaling pathways in the ovaries, fat body and midgut (Hansen et al., 2004; Zhu et al., 2003; Zhu et al., 2006; Clifton and Noriega, 2011). Oogenesis remains arrested, however, until a blood meal is taken, which triggers the release of insulin-like peptides (ILPs) and ovary ecdysteroidogenic hormone (OEH) from neurosecretory cells in the brain (Riehle et al., 2006; Brown et al., 1998). ILP3, ILP4 and OEH stimulate the ovaries to produce ECD (Brown et al., 1998; Brown et al., 2008; Wen et al., 2010). ILP3 together with amino acid sensing through the TOR pathway induces serine protease activity in the midgut that digests the blood meal, while ILP3, TOR and ECD interact to upregulate the expression of vitellogenin (VG)

and other yolk proteins by the fat body (Hansen et al., 2005; Roy et al., 2007; Bryant et al., 2010; Roy and Raikhel, 2011; Gulia-Nuss et al., 2011). Packaging of yolk proteins into oocytes followed by chorion formation then results in the formation of 120–150 mature eggs, which females then oviposit.

The rockpool mosquito, *Georgescraigius atropalpus* (Coquillett 1902) (formerly *Aedes* and *Ochlerotatus*) (Culicinae, Aedini), resides in the same tribe as *A. aegypti* (Reinert et al., 2009) but is facultatively autogenous. Prior studies indicate that ECD production by the ovaries and yolk uptake by oocytes rise and fall within 10–50 h post-eclosion (PE) followed by oviposition at 60–72 h PE (Fuchs et al., 1980; Masler et al., 1980; Masler et al., 1981; Birnbaum et al., 1984; Kelly et al., 1981; Kelly et al., 1984). Decapitation of *G. atropalpus* females immediately after eclosion blocks yolk uptake into oocytes, while injection of crude head extracts from *A. aegypti* and 20-hydroxyecdysone (20E) partially rescue egg maturation. To mature a second clutch of eggs, *G. atropalpus* requires a blood meal, which females seek approximately 7 days after laying the first clutch of eggs (Hudson, 1970; Masler et al., 1983; Bowen et al., 1994). Taken together, these results suggest that, similar to *A. aegypti*, egg maturation by *G. atropalpus* depends upon neuroendocrine factors from the brain. The identity of these neuroendocrine factors, however, and whether the same cascade of events occurs in producing a blood meal-independent first clutch or blood meal-dependent second clutch is unknown. Here, we present results indicating that OEH is the key factor released from the brain that stimulates both first and second egg clutch formation in *G. atropalpus*.

MATERIALS AND METHODS

Mosquitoes

Georgescraigius atropalpus (Bass Rock strain, founded from a colony at the University of Arizona) was maintained as described previously (Telang and Wells, 2004; Telang et al., 2006). Eggs for colony maintenance were obtained from first clutches laid by females fed a 10% sucrose solution. For mating studies, late pupae (*ca.* 24 h prior to adult eclosion) were sexed by size, and the larger female pupae held separately from male pupae in cages for adult emergence. Mated females were collected from cages holding the same cohort of both sexes. All females mated shortly after eclosion as confirmed by the observation of sperm in the spermatheca. Rats were anesthetized and provided to females for blood feeding in compliance with the Animal Care and Use Program at the University of Georgia.

Reagents

Aedes aegypti ILP3 (AaILP3) and ILP4 (AaILP4) were synthesized as previously described (Brown et al., 2008; Wen et al., 2010). 20E (Sigma) was solubilized in absolute ethanol. Recombinant *A. aegypti* OEH (AarIOEH; residues 23–149, minus the signal peptide) was produced in *Escherichia coli* and purified as previously outlined (Brown et al., 1998) with the exception that OEH was cloned into pET-32 instead of pET-17. A stock of the AarIOEH (100–150 pmol μl^{-1}) in water was prepared for dilution prior to bioassay. The dose-responsive bioactivity of AarIOEH (13.3 kDa) was equivalent to that of the native truncated form (8803 kDa) isolated from head extracts, as determined by gonadotropic bioassays with *A. aegypti* (Brown et al., 1998).

First clutch *in vivo* assays

Newly eclosed females (0–6 h PE) were decapitated and injected with AarIOEH, AaILPs or 20E, in 0.5 μl of saline. Decapitated females injected with saline served as a negative control while intact (non-decapitated) females served as a positive control. Decapitated

females were then held in small cages housed in humidified chambers while intact females were held similarly but with access to water. For oviposition assays, at least 10 females per treatment were kept individually in small cages lined with a moist paper towel, and eggs were counted 96–120 h PE. Tissues were dissected from females in saline and set up as triplicate samples for the assays described below. Experiments were replicated with at least three different cohorts, and the following data were collected.

Yolk deposition was determined by measuring the length of the anterior–posterior axis of 10 oocytes per ovary pair of each experimental female. ECD production by ovaries *in vitro* (2 pairs/dish) was assayed after incubation for 6 h (27°C) in 60 μl of saline or amino acid-rich medium (Sf-900 II SFM medium; cat. no. 10902, Gibco, Gaithersburg, MD, USA) alone or with other reagents. ECD content in each sample of medium (50 μl) was determined by radioimmunoassay (RIA) (Wen et al., 2010). Total protein was determined for abdomen walls (i.e. whole-abdomen body wall with attached fat body minus gut and ovaries) and hemolymph at different times during egg maturation. Two females were opened in 100 μl of saline, and the diffused hemolymph was transferred to a tube. The abdomen walls were dissected free from both females, homogenized in saline and centrifuged (12,000 g, 4°C). After appropriate dilution, the protein concentration of abdomen wall supernatant and hemolymph samples was determined by the Bradford assay (Pierce, Rockford, IL, USA). Serine protease activity was assayed in abdomen wall, thorax and midgut samples (Gulia-Nuss et al., 2011). To determine trypsin-like activity, 2 tissues/sample were transferred to 200 μl of 20 mmol l^{-1} Tris (pH 8.0 with 20 mmol l^{-1} CaCl_2), sonicated and centrifuged (14,000 g, 2 min). Supernatants were frozen (–80°C), and later 0.05 tissue/10 μl was added to 200 μl of 4 mmol l^{-1} *N*- α -benzoyl-L-arginine-*p*-nitroanilide (BAPNA) in a well of a 96-well plate for 10 min followed by measurement of absorbance at 405 nm (Biotek plate reader, Winooski, VT, USA). Enzyme activity was then calculated from a regression line formula using trypsin standards (bovine pancreas, Sigma T1426, St Louis, MO, USA). To assay chymotrypsin-like activity (Masler et al., 1983), tissues were transferred to 200 μl of 0.1 mmol l^{-1} cold HCl and processed as above. Later, 0.05 tissue/sample was added to a well with 140 μl of 20 mmol l^{-1} Tris (pH 7.0) and 8 μl 20 mmol l^{-1} CaCl_2 , followed by 100 μl benzoyl-L-tyrosine ethyl ester (BTEE) substrate. Samples were incubated for 5 min, loaded in wells of a Greiner UV-compatible 96-well plate for measurement of absorbance at 256 nm (Biotek plate reader). Activity was quantified based on α -chymotrypsin standards (bovine pancreas, Sigma C4129).

Second clutch *in vivo* assays

After oviposition of the first egg clutch, females were maintained in cages and provided with a sucrose solution *ad libitum*. At 10–12 days PE, females were attracted to a human arm held near the cage, and only then were females given access to an anesthetized rat for blood feeding. Only females that took a full blood meal were used in the experimental procedures and assays as described above. Bioassays as above were replicated with at least three different cohorts.

In vitro assays

Females decapitated <6 h PE were held overnight in small cages in a humidified chamber (27°C). Abdomen walls (2 mosquitoes cut along one side to float flat on 100 μl) and midguts (2 in 60 μl) were incubated 4 h (27°C) in saline or Sf-900 II SFM medium alone or with AarIOEH, AaILPs or 20E and then assayed for serine protease

activity or yolk deposition into oocytes, as described above. Ovaries (2 pairs in 60 μ l) were similarly incubated for 6 h and assayed for ECD production. Triplicate tissue samples were prepared for each of three different cohorts.

RNA interference knockdown of the *G. atropalpus* insulin receptor

Total RNA was isolated from *G. atropalpus* abdomen walls and first strand cDNA was synthesized as previously described (Gulia-Nuss et al., 2011). A portion of the *G. atropalpus* insulin receptor (IR) gene was amplified from this cDNA pool using specific primers (forward 5'-TAATACGACTCACTATAGGGCCGGAGGTGAA-TCCAGACTA-3' and reverse 5'-TAATACGACTCACTATA-GGGCTTCTTTGCCGAAAGTACGC-3') for the corresponding carboxyl domain (nucleotides 3001–3546) of the *IR* gene from *A. aegypti* (Brown et al., 2008). The purified PCR product (Qiaprep, Qiagen, Valencia, CA, USA) was cloned into TOPO-TA, sequenced, and determined to be 95% identical to the *A. aegypti* *IR* (CLUSTAL 2.0.12). We therefore prepared dsRNA corresponding to nucleotides 3079–3521 of the *A. aegypti* *IR* and an enhanced green fluorescent protein (eGFP) dsRNA control as previously described (Gulia-Nuss et al., 2011). Intact or decapitated *G. atropalpus* females (<6 h PE) were then injected with 2 μ g of each dsRNA in 0.5 μ l water. Knockdown of the *G. atropalpus* *IR* was then determined by RT-PCR amplification of actin and *IR* products from cDNA processed from abdomen walls of females 24 h post-dsRNA injection (Gulia-Nuss et al., 2011). Bioassays as above were replicated with at least three different cohorts.

Immunoblots

To detect VG, abdomen walls were dissected in saline and transferred to sample buffer for sonication/extraction and centrifugation. Supernatants were loaded on a 4–20% Tris-HCl gel (BioRad Criterion gels, Hercules, CA, USA), transferred to nitrocellulose membrane (Protran 0.2 μ m, Whatman), and probed with rabbit antiserum to *A. aegypti* VG (R2,1; 1:100,000 dilution) (Gulia-Nuss et al., 2011). Immunoreactive proteins were visualized with a peroxidase-conjugated goat anti-rabbit secondary antibody (1:20,000 dilution; Sigma A6667) and chemiluminescent substrate (ECL Advance kit, GE Healthcare, Wauwatosa, WI, USA). To detect OEH, heads were removed from females, and hemolymph collected as above in saline and 2 \times tricine sample buffer. Samples were sonicated and centrifuged, and the supernatants loaded on 16% Tris-tricine gels (BioRad Criterion gels). After transfer to nitrocellulose membrane (Protran 0.1 μ m, Whatman) and drying, the blot was incubated with rabbit antiserum to *A. aegypti* OEH (304C, 1:10,000 dilution) (Brown and Cao, 2001).

Data analysis

All analyses were conducted using the JMP 7.0 statistical platform (SAS, Cary, NC, USA). Yolk deposition into oocytes, ecdysteroid production by ovaries, and serine protease activity were analyzed by ANOVA followed by the Tukey–Kramer multiple comparison procedure with treatment serving as the independent variable. The number of mature follicles and number of eggs laid by virgin and mated females were analyzed by *t*-test.

RESULTS

OEH and 20E stimulate production of the first autogenous egg clutch

Given the roles of OEH and ILPs in egg maturation by *A. aegypti*, we first asked whether decapitation of newly emerged *G. atropalpus*

blocked egg maturation and whether OEH or two ILPs from *A. aegypti* rescued this response. Our results showed decapitated females deposited no yolk into eggs while a single injection of AarIOEH (10 pmol) or 20E (1 μ g) rescued yolk deposition to levels seen in intact females at 24 and 48 h PE (Fig. 1A). A single dose of AaILP3 (20 pmol) stimulated only half the level of yolk deposition observed in intact females, while the same dose of AaILP4 had no stimulatory effect (Fig. 1A). Higher doses of each ILP did not alter these outcomes (data not shown). AarIOEH and 20E but not ILPs also rescued VG synthesis in the fat body (abdomen wall) and ECD production by the ovaries (Fig. 1B, top and bottom panel, respectively).

Production of the first egg clutch by *G. atropalpus* is dependent on mobilization of the general protein reserve in the fat body by chymotrypsin-like serine protease activity, as shown in a previous study (Masler et al., 1983). Consistent with this finding, we observed that chymotrypsin-like activity increased in the fat body of intact females but not in decapitated ones by 24 h PE (Fig. 1C). Neither chymotrypsin-like activity nor trypsin-like serine protease activity was detected in the midguts of the experimental females (Fig. 1C; also not in other tissues, data not shown). AarIOEH and 20E but not AaILP3 restored this chymotrypsin-like activity in decapitated females to the level in intact females (Fig. 1C). Fully consistent with this increase in protease activity, AarIOEH and 20E treatment also reduced total protein content in the fat body and increased hemolymph protein to levels observed in intact females (Fig. 1D), thus reflecting fat body secretion of VG into hemolymph. In control decapitated females, the high protein content in fat body and low hemolymph protein indicate VG secretion is not activated (Fig. 1D).

Georgacraigius atropalpus releases OEH from the brain between 4 and 8 h PE

As we used AarIOEH in the preceding assays, we assessed whether *G. atropalpus* produces OEH by probing immunoblots of head extract and hemolymph with our AaOEH antiserum. AaOEH (13.7 kDa processed long form) was readily detected in *A. aegypti* head extract, as was AarIOEH (Fig. 2). A corresponding 14 kDa band was detected in head extracts from *G. atropalpus* pupae and adult females at different times PE (Fig. 2). We also detected the putative native OEH in the hemolymph of adult females at 8 h and 24 h PE, which coincides with the activation of egg maturation for the first clutch (Fig. 2). It was not present in the hemolymph of pupae or females at 4 h PE, thus suggesting release from the brain neurosecretory cells occurs around 6 h PE, the latest time for decapitation to prevent autogeny.

OEH and 20E activate different tissue-specific processes *in vitro*

The preceding results indicated that AarIOEH was more bioactive than AaILPs in stimulating ECD production, VG synthesis and mobilization of nutrient stores. To confirm that the effects of these neuropeptides on the ovary and fat body were direct, we conducted *in vitro* assays using primary cultures. We also compared the effect of each neuropeptide when each tissue was cultured in saline *versus* amino acid-rich Sf-900 II SFM medium (AA medium) because the latter greatly enhances the *in vitro* response of *A. aegypti* tissues (Gulia-Nuss et al., 2011). Preliminary studies indicated that ovaries and fat bodies isolated at 6 h PE from *G. atropalpus* females were not responsive to either OEH or ILPs, but those taken from females decapitated and held overnight were fully responsive. AarIOEH and AaILP3 stimulated ovaries to produce significantly more ECD than control ovaries, whereas AaILP4 had no stimulatory effect (Fig. 3A).

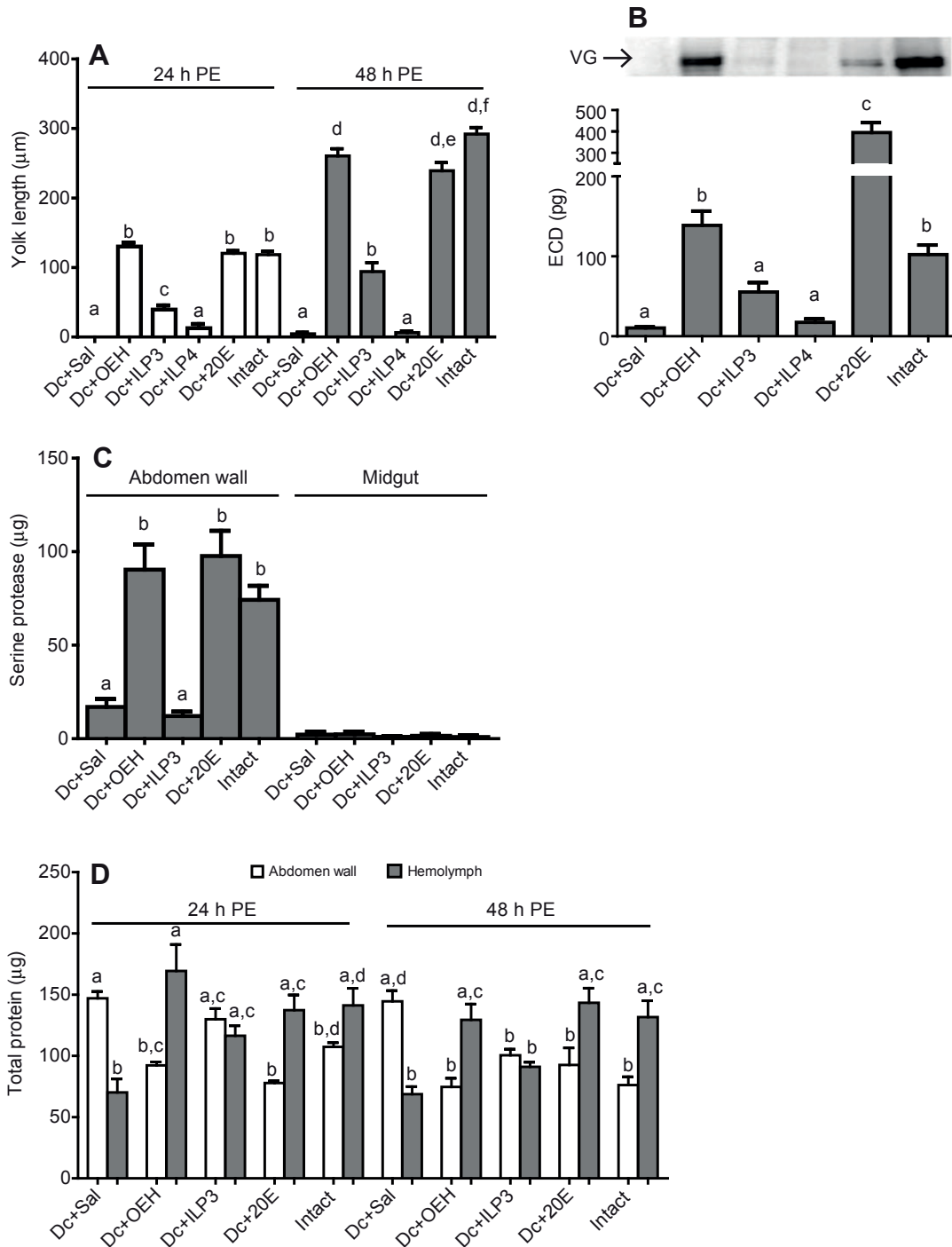


Fig. 1. *Aedes aegypti* recombinant long ovary ecdysteroidogenic hormone (Aar/OEH) and 20-hydroxyecdysone (20E) restore autogenous egg maturation in decapitated *Georgaeraigius atropalpus* females. (A) Decapitation (Dc) of females at 6 h post-eclosion (PE) blocks yolk deposition in oocytes (Dc+Sal, negative control), but a single injection of Aar/OEH (10 pmol, OEH) or 20E (1 µg) after decapitation stimulates yolk deposition (mean ± s.e.m. yolk length/oocyte) to the same extent as in intact females at 24 and 48 h PE ($F_{11,390}=175.4$, $P<0.0001$). *Aedes aegypti* insulin-like peptide 3 (AaILP3, 20 pmol, ILP3) is less effective and AaILP4 (20 pmol, ILP4) has no activity. Intact females injected with saline (Sal) served as positive controls. Different letters above bars in the graph indicate means that significantly differ; comparison of all pairs of means was made using the Tukey–Kramer procedure, $\alpha=0.05$ (see Materials and methods). (B) Aar/OEH stimulates vitellogenin (VG) expression in fat body and ecdysteroid hormone (ECD) production in ovaries of decapitated females to the same levels detected in intact females. Top, representative immunoblot of VG (220 kDa) expression at 24 h PE in abdomen walls (0.05 wall/lane) of females treated as above. Bottom, ECD production by ovaries (mean and s.e.m. for 2 pairs/6 h) taken at 24 h PE from females treated as above ($F_{5,120}=50.8$, $P<0.0001$). (C) Aar/OEH and 20E induce chymotrypsin-like serine protease activity (BTEE substrate; mean and s.e.m. values per abdomen) in the abdomen wall of decapitated females to the same level in intact females at 24 h PE ($F_{4,31}=26.6$, $P<0.0001$). No chymotrypsin-like activity detected in midguts from the same females (mean and s.e.m. values per midgut). (D) Aar/OEH and 20E treatment of decapitated females reduces teneral protein in abdomen walls and increases hemolymph protein at 24 and 48 h PE (mean and s.e.m. values per female). Corresponding levels in intact females indicate yolk protein secretion, but reciprocal levels in decapitated control females reflect inhibition of egg maturation ($F_{19,173}=10.2$, $P<0.0001$).

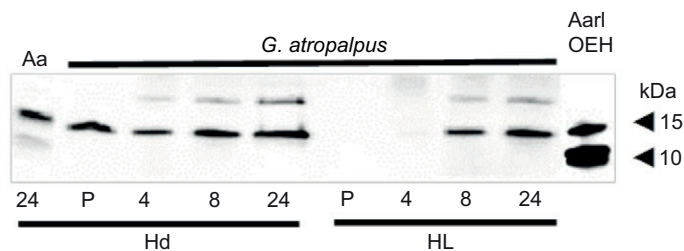


Fig. 2. Representative immunoblot of head extracts and hemolymph probed with AaOEH antiserum. Left side shows head extracts (Hd, 4 heads/lane) from female *A. aegypti* (Aa, positive control) and *G. atropalpus* pupae (P) and females at 4, 8 and 24 h PE. Right side shows hemolymph (HL) collected from *G. atropalpus* pupae and females at 4, 8 and 24 h PE (20 females/lane) and AarIOEH (5 pmol/lane, positive control). Mass markers for 10 and 15 kDa are indicated on the right.

The absolute response to each neuropeptide, however, was also significantly higher for ovaries cultured in AA medium than in saline, suggesting a key role for amino acid sensing in this response. AarIOEH significantly increased chymotrypsin-like activity in fat bodies incubated with or without AA medium (Fig. 3B), but no increase in activity was detected in response to ILPs or 20E. In contrast, only 20E stimulated VG synthesis *in vitro* with a stronger response detected in fat bodies cultured in AA medium than in saline (Fig. 3C).

OEH activity does not depend on the insulin receptor

OEH and ILP3 exhibit similar gonadotropic activity in *A. aegypti* (Brown et al., 1998; Brown et al., 2008), thus suggesting that, like ILP3, OEH may interact with the mosquito IR. Our sequencing of *G. atropalpus* IR revealed that it is nearly identical to that of *A. aegypti* (see Materials and methods). However, as AarIOEH exhibits much greater activity than AaILP3 in *G. atropalpus*, we reasoned this semi-autogenous species could provide interesting insights into whether OEH activity is IR dependent or independent.

We used RNA interference (RNAi) to knock down expression of *G. atropalpus* IR. The absence of IR expression was confirmed by RT-PCR for decapitated and intact females 24 h after injection of IR and *eGFP* dsRNA (Fig. 4A). We then compared the ability of AarIOEH and AaILP3 to restore yolk deposition into oocytes and ovary ECD biosynthesis in decapitated, IR knockdown females relative to control (*eGFP* dsRNA pretreated), decapitated females. Strikingly, our results showed that AarIOEH rescued both responses in IR knockdown females to levels similar to those observed in control females (Fig. 4B,C). In contrast, ILP3 had essentially no effect in IR knockdown females relative to its activity in control females.

We also examined the effect of IR knockdown on the same processes in intact *G. atropalpus* females. Control (*eGFP* dsRNA pretreated) females injected with saline deposited the same amount of yolk in oocytes as normal, intact *G. atropalpus* (Fig. 4D; see Fig. 1A for intact level). Injection of AarIOEH or AaILP3 into cohort control females also did not alter this process (Fig. 4B,C), and presumably did not interfere with the release of endogenous OEH and ILPs from their brains. Ovary ECD production was low in the control females injected with saline (Fig. 4E) because this process is normally completed by 48 h PE (the assay time), but injection of AarIOEH and AaILP3 extended ECD production in the control females (Fig. 4E). In contrast, the IR knockdown females injected with saline or AaILP3 deposited almost no yolk into oocytes and exhibited low ECD production (Fig. 4D,E), but injection of AarIOEH into these females restored both processes to levels similar to those in the control females injected with the two peptides (Fig. 4D,E). Taken together, these data

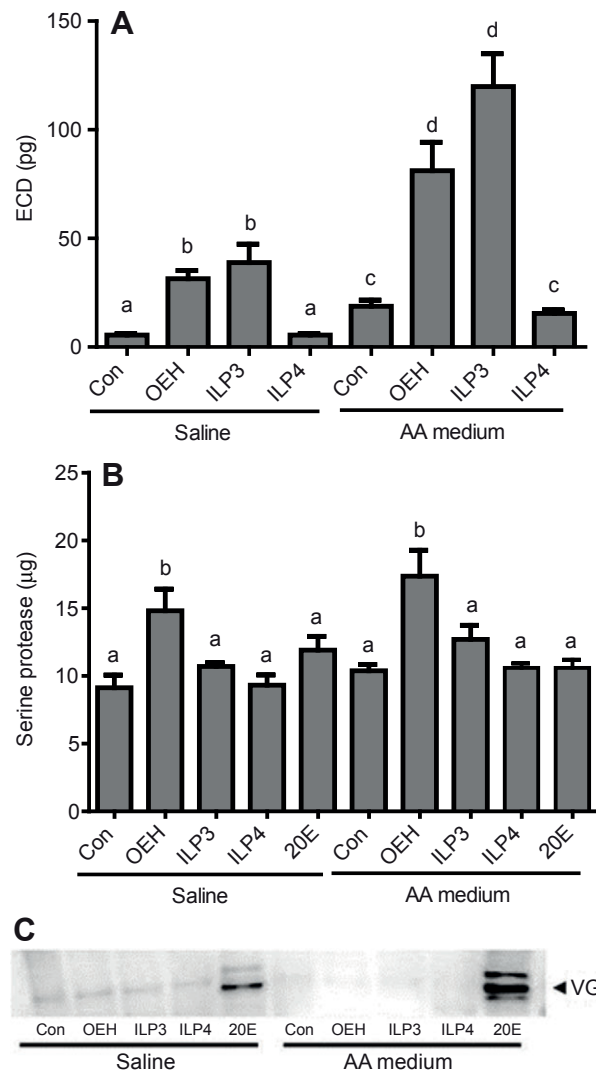


Fig. 3. AarIOEH, AaILP3 and 20E activate different tissue-specific processes *in vitro*. *Georgacraigius atropalpus* females were decapitated at <6 h PE and held overnight. Dissected ovaries or abdomen walls were incubated in saline (Sal) or amino acid-rich Sf-900 II SFM medium (AA medium) alone (control, Con) and with the reagents. Statistical comparisons were performed as indicated in Fig. 1A. (A) Ovary ECD production (mean and s.e.m. for 2 pairs/6 h) is stimulated by AarIOEH (10 pmol, OEH) and AaILP3 (20 pmol, ILP3), but not AaILP4 (20 pmol, ILP4) (Saline: $F_{3,35}=12.6$; AA: $F_{3,38}=22.8$; $P<0.0001$). AA medium enhances activation of this process. (B) Chymotrypsin-like serine protease activity (BTEE substrate; mean and s.e.m. values per abdomen for 4 h) is induced by AarIOEH (15 pmol) but not by the AaILPs (30 pmol) or 20E (1.5 µg) ($F_{9,30}=6.4$, $P<0.0001$). (C) Representative immunoblot showing induction of VG (220 kDa) synthesis in abdomen walls (0.5 wall/lane) incubated with 20E (1.5 µg) but not in samples incubated with AarIOEH (15 pmol) or the AaILPs (30 pmol).

strongly suggested that AarIOEH activity in *G. atropalpus* does not depend on the IR, whereas AaILP3 activity does.

Maturation of eggs in the first clutch does not depend upon mating

As *G. atropalpus* produces a first clutch without blood feeding, we asked whether mating served as an alternative signal for stimulating the release of OEH and ILPs from the brain of newly emerged females. Our results showed that yolk deposition into oocytes did

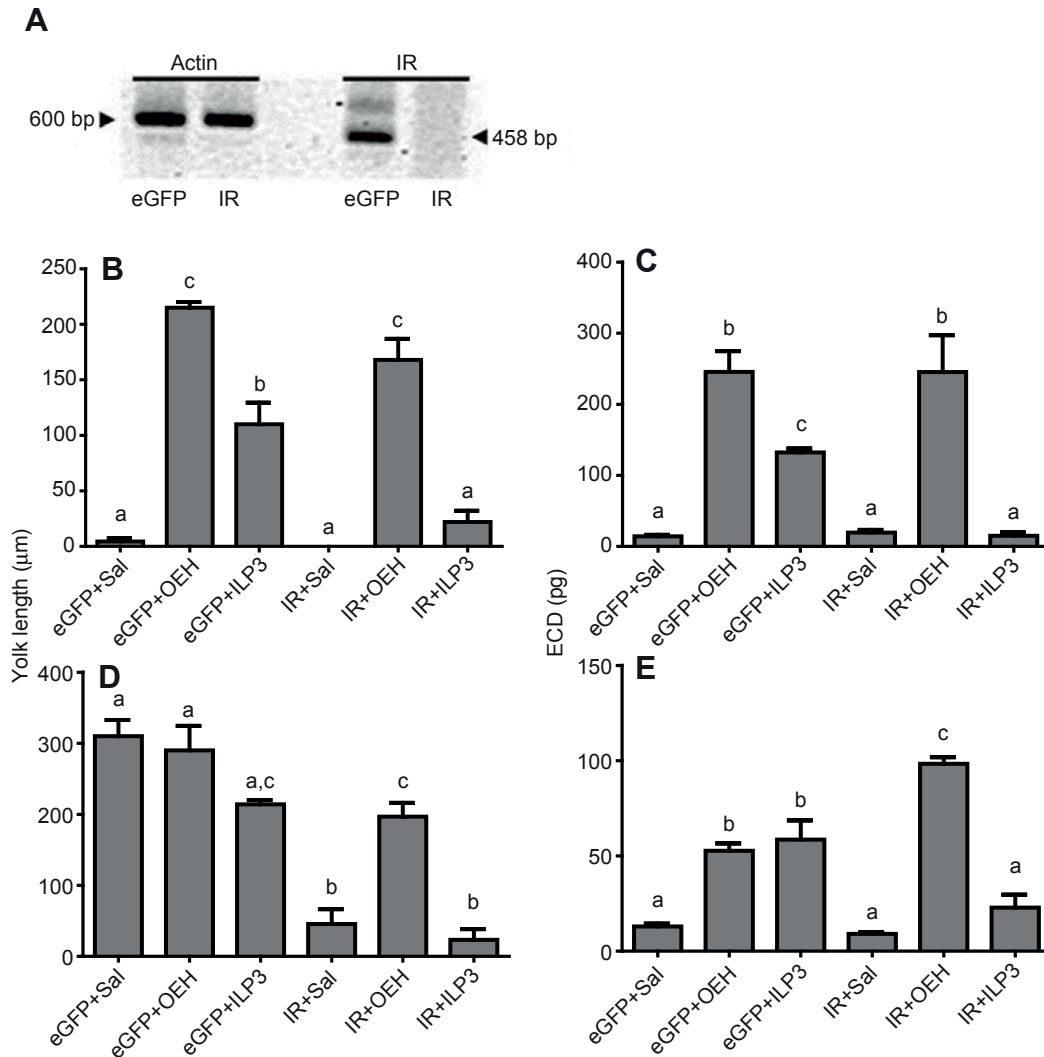


Fig. 4. OEH-mediated yolk deposition and ECD production does not require the insulin receptor (IR) in *G. atropalpus*. Females were injected with *IR* or enhanced green fluorescent protein (*eGFP*, control) dsRNA at <6 h PE, then decapitated or left intact, held overnight and injected with saline (control), AarIOEH (10 pmol) or AaILP3 (20 pmol). After 24 h or approximately 48 h PE, tissues were assayed as below. Statistical comparisons were performed as indicated in Fig. 1A. (A) *Georgacraigius atropalpus IR* expression is silenced by *IR* RNAi. RT-PCR of actin and *IR* products amplified from cDNA processed from abdomen walls of females 24 h post-dsRNA injection. (B) Oocyte yolk deposition (mean and s.e.m. yolk length/oocyte) in decapitated females ($F_{5,46}=56.7$, $P<0.0001$). (C) ECD production by ovaries (mean and s.e.m. for 2 pairs/6 h) taken from decapitated females ($F_{5,33}=24.0$, $P<0.0001$). (D) Oocyte yolk deposition (as above) in intact females ($F_{5,47}=23.5$, $P<0.0001$). (E) ECD production by ovaries (as above) taken from intact females ($F_{5,26}=39.3$, $P<0.0001$).

not differ greatly between mated and virgin females at 24, 48 or 72 h PE (Fig. 5A). The number of follicles (~60/ovary) with mature oocytes also did not differ (data not shown). However, mating status was crucial for oviposition as mated females laid more than 100 eggs by 120 h PE, whereas virgin females laid almost none (Fig. 5B). Subsequent observations found that virgin females retained mature oocytes for at least 10 days.

OEH and 20E also stimulate production of a second egg clutch

As *G. atropalpus* fully depends on blood feeding to produce a second egg clutch, we assessed whether the stimulatory activity of AarIOEH, AaILPs and 20E in egg maturation remained similar to or differed from the first clutch. The females of our *G. atropalpus* colony are not attracted to a human host for approximately 7 days after ovipositing the first clutch of eggs. Thereafter, females would orient

to a human host and feed from an anesthetized rat. Decapitation of females within 1 h post-blood meal blocked egg maturation, while a single injection of AarIOEH (10 pmol/female) or 20E (1 μg) rescued yolk deposition into oocytes, VG synthesis by the fat body, and ECD production by the ovaries to levels observed in intact females at 24 h post-blood meal (Fig. 6A,B). Similar to outcomes in producing a first clutch, AarIOEH stimulated more yolk deposition and VG synthesis than AaILP3, while AaILP4 exhibited no activity. Also similar to production of a first clutch, AarIOEH and 20E stimulated ECD production by the ovaries but neither ILP exhibited any activity.

Whereas chymotrypsin-like activity rises in the fat body during first-clutch formation, an earlier study found that trypsin-like serine protease activity is predominant in the whole body of *G. atropalpus* 12–48 h post-blood feeding (Masler et al., 1983). Our assays revealed that the trypsin-like activity is restricted to the midgut of

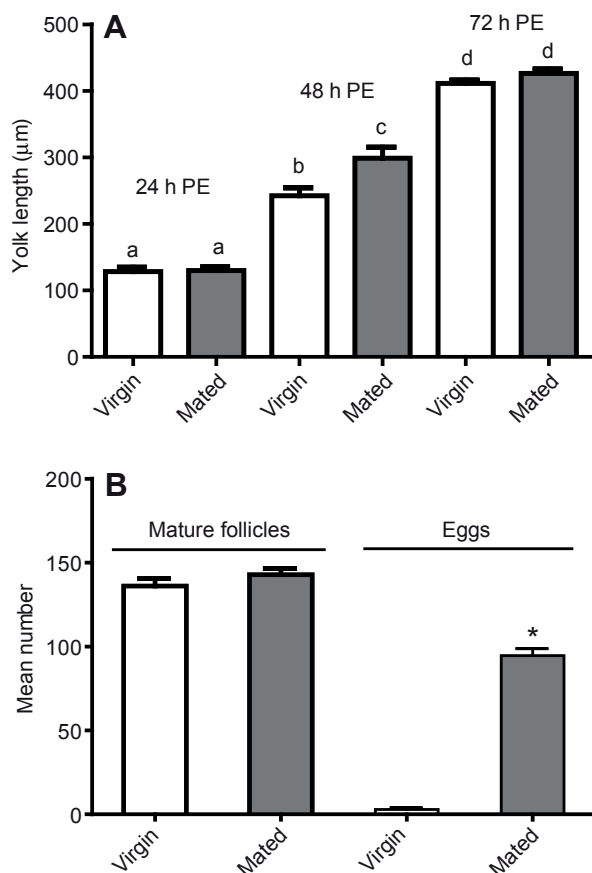


Fig. 5. Mating affects egg laying by *G. atropalpus* but not autogenous egg maturation. (A) Oocyte yolk deposition (mean and s.e.m. yolk length/oocyte) in virgin and mated females at 24, 48 and 72 h PE ($F_{5,97}=188.9$, $P<0.0001$). Statistical comparisons performed as indicated in Fig. 1A. (B) Mature follicles (observed at 72 h PE) and eggs oviposited by virgin and mated females (mean and s.e.m. number/female). No difference was observed in follicle numbers, but mated females laid significantly more eggs (*) than virgin females (t -tests, $\alpha\leq 0.01$, $N=16-20$ females per treatment).

intact blood-fed females (Fig. 6C; data for other tissues not shown), and no chymotrypsin-like activity is detected in the midgut or other tissues of these females (data not presented). Decapitation after blood feeding blocked the increase in trypsin-like activity observed in intact females at 24 h post-blood meal, but a single dose of AarIOEH restored this to the same level of activity in decapitated females (Fig. 6C). In contrast, neither AaILPs or 20E had any rescue effect (Fig. 6C).

DISCUSSION

Vertebrate blood provides a rich source of nutrients for egg production by mosquitoes, but acquisition of this resource also has costs that include the time required to find a suitable host and the risk of mortality while feeding. The ability to produce eggs without blood feeding obviously eliminates these costs, but the physiological and/or regulatory changes that underlie the evolution of autogeny largely remain unclear. In the case of *G. atropalpus*, females always produce their first clutch of eggs autogenously while a second clutch requires a blood meal (Hudson, 1970; Masler et al., 1983; Bowen et al., 1994). The amount of food a female acquires during the larval stage does not alter this pattern, although teneral reserves and sugar feeding as an adult do affect how many eggs a female produces in

her first clutch (i.e. clutch size) (Telang and Wells, 2004; Telang et al., 2006). Genetic analysis indicates that autogeny is monofactorial and not sex linked (O'Meara and Craig, 1969; Gwadz, 1970; O'Meara and Krasnick, 1970; O'Meara, 1972; Masler et al., 1981). Prior studies also clearly establish that maturation of a first clutch depends on the release of neurohormones from the brain, which stimulates ECD production by the ovaries, mobilization of teneral nutrient reserves in the fat body, and VG production (Van Handel, 1976; Fuchs et al., 1980; Masler et al., 1980; Masler et al., 1981; Masler et al., 1983; Birnbaum et al., 1984; Kelly et al., 1981; Kelly et al., 1984; Ma et al., 1984).

The central question of this study was whether OEH and ILPs, which regulate egg maturation in *A. aegypti*, also regulate egg production in *G. atropalpus*. Our results show that a single dose of AarIOEH stimulates maturation of both a first and a second clutch in decapitated females by restoring ovary ECD production and protein mobilization, so that vitellogenesis and yolk deposition can proceed. AaILP3, in contrast, is less effective in rescuing maturation of either clutch, while AaILP4 exhibits no activity in *G. atropalpus*. Both AarIOEH and AaILP3 directly stimulate ovaries of *G. atropalpus* to produce ECD with the activity of each being greater in medium containing amino acids. This finding is very similar to previous studies with *A. aegypti* which showed that ILP/insulin signaling interact with amino acid sensing through the TOR pathway to activate ovary ECD production and VG synthesis (Hansen et al., 2005; Roy et al., 2007; Roy and Raikhel, 2011; Gulia Nuss et al., 2011). Prior studies show that *G. atropalpus* ovaries predominantly produce ecdysone, which is converted to 20E in target tissues, and that 20E stimulates egg maturation in decapitated autogenous females (Masler et al., 1980; Birnbaum et al., 1984). Our study confirmed that 20E activates maturation not only of the first egg clutch but also of the second one in decapitated *G. atropalpus* females, and it alone stimulates VG synthesis in isolated *G. atropalpus* fat body. These findings are fully shared with *A. aegypti* (Attardo et al., 2005; Roy et al., 2007) in that ECD signaling is a potent promoter of VG synthesis. Finally, our results strongly suggest that AarIOEH mimics a native OEH detected in the brains of *G. atropalpus* females and released into the hemolymph at the initiation of autogenous egg maturation.

Taken together, our results indicate that, as in *A. aegypti*, OEH, ILP, ECD and amino acid/TOR signaling control egg maturation in *G. atropalpus* during formation of both an autogenous first clutch and anautogenous second clutch. The crucial difference is that *G. atropalpus* produces an autogenous first clutch because it releases OEH and likely ILPs within the first 6–12 h after eclosion without blood feeding, whereas OEH and ILP release in *A. aegypti* is blood meal dependent. Transplantation experiments conducted with autogenous and anautogenous strains of the salt marsh mosquito, *Aedes taeniorhynchus*, suggest that blood meal-independent release of neurohormones from the brain also underlies autogenous production of eggs (Lea, 1970). Notably, OEH/ILP release by *G. atropalpus* for production of a second clutch is blood meal dependent, suggesting that regulation of OEH/ILP release changes in females over time. Other key transitions to enable blood feeding include the shift from fat body to midgut for enzymatic mobilization of amino acids for VG synthesis and the acquisition of host-seeking behavior (Bowen et al., 1994). The regulation of these changes may involve some combination of these signaling elements and novel ones that similarly underpin variations in autogeny/anautogeny observed in mosquitoes (O'Meara, 1985).

Given that all mosquito genomes examined to date (*A. aegypti*, *Anopheles gambiae* and *Culex pipiens*) encode an OEH and multiple

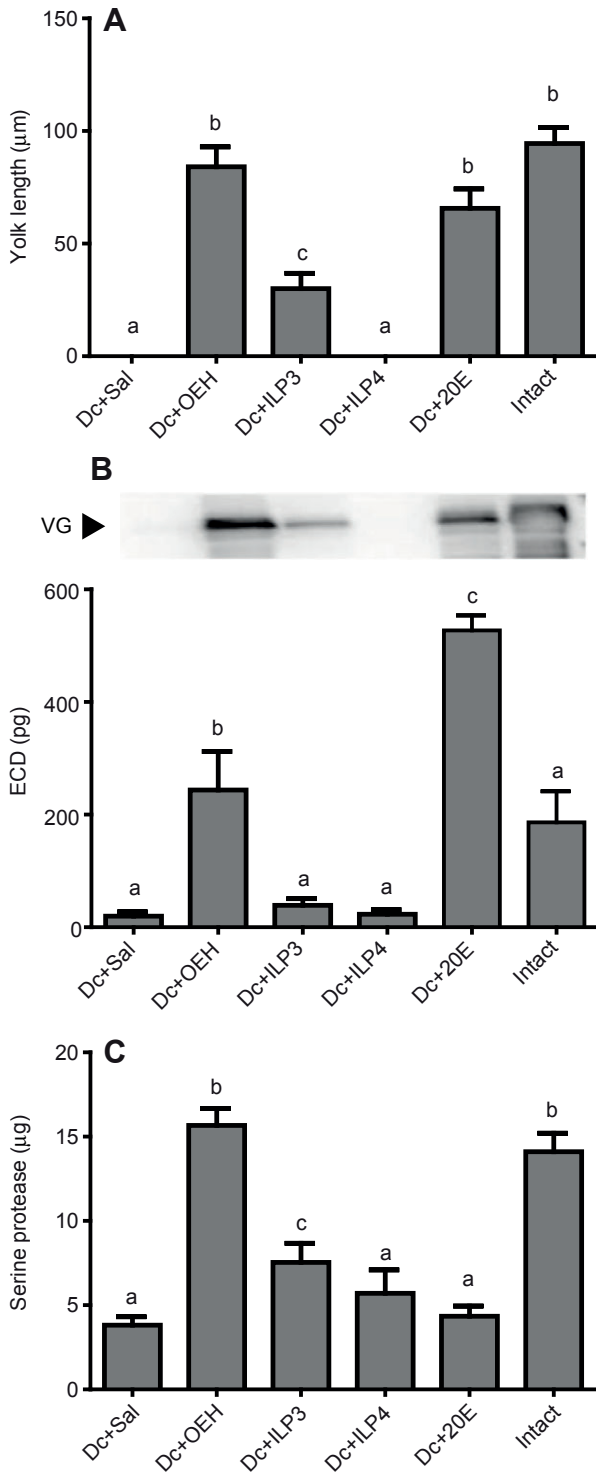


Fig. 6. AarIOEH and 20E restore egg maturation in blood-fed, decapitated female *G. atropalpus*. At 12 days PE, females were blood fed, decapitated (Dc) after 1 h, and given a single injection of saline (Sal, negative control), AarIOEH (10 pmol, OEH), AaILP3 (20 pmol, ILP3), AaILP4 (20 pmol, ILP4) or 20E (1 μg). Intact females were injected with saline as a positive control. At 24 h post-blood meal, females were processed for the following assays. Statistical comparisons were performed as indicated in Fig. 1A. (A) AarIOEH and 20E stimulate yolk deposition (mean and s.e.m. yolk length/oocyte) in decapitated females to levels observed in intact females ($F_{5,159}=37.6$, $P<0.0001$). (B) AarIOEH and 20E stimulate VG expression in fat body and ECD production in ovaries of decapitated females. Top, representative immunoblot of VG (220 kDa) in abdomen walls (0.05 wall/lane). Bottom, ECD production by ovaries (mean and s.e.m. for 2 pairs/6 h) ($F_{5,53}=16.9$, $P<0.0001$). (C) AarIOEH induces trypsin-like serine protease activity (BApNA substrate; mean and s.e.m. values per midgut) in the midgut of decapitated females to the same level as that in intact females ($F_{5,127}=30.1$, $P<0.0001$).

corpus cardiacum, which is innervated by the medial neurosecretory cell axons (Brown and Cao, 2001; Riehle et al., 2006). Studies with *A. aegypti* indicate that JH released from the corpora allata after eclosion but prior to a blood meal establishes tissue competency to respond to OEH/ILP signaling by promoting expression of the TOR and ECD signaling pathways (Li et al., 2003; Shiao et al., 2008; Clifton and Noriega, 2011). The results of our *in vitro* assays likewise suggest that tissues from *G. atropalpus* must acquire competency to respond to OEH. However, JH has no known role in stimulating the synthesis or the release of OEH and ILPs in *A. aegypti* or any other mosquito.

Other factors suggested to affect autogenous egg production include teneral nutrient reserves, sugar feeding and mating (Hudson, 1970; O'Meara and Krasnick, 1970; Kalpage and Brust, 1974; O'Meara, 1985; Wheeler and Buck, 1996; Telang and Wells, 2004; Telang et al., 2006). As previously noted, differences in teneral reserves and sugar intake clearly affect first clutch size by determining the amount of VG and other yolk proteins a female can produce. Suboptimal teneral reserves or sugar intake, however, never affect the ability of *G. atropalpus* females to always produce their first clutch autogenously. These findings collectively suggest nutrient availability is not the sole determinant of OEH/ILP release, although it is possible that nutrient stores or a particular level of some factor, like amino acids, plays a role in this process. In contrast, our results strongly indicate that mating status does not affect OEH/ILP release because virgin and mated females mature a first clutch of eggs at exactly the same time after emerging. In contrast, mating does profoundly affect oviposition by *G. atropalpus* given that mated females lay most or all of the eggs they mature while virgin females lay almost none. Clearly then a key goal for future studies will be to identify the factor(s) that triggers the release of OEH and ILPs in anautogenous species like *A. aegypti*. Another will be to determine whether autogenous species like *G. atropalpus* rely on the same factor(s) but control its release independently of blood feeding or regulate OEH/ILP release entirely differently.

We recognize the lesser activity of AaILP3 in *G. atropalpus* may be that it suboptimally mimics the endogenous ILP(s) in *G. atropalpus*, given that all insects including mosquitoes encode multiple ILP family members (Marquez et al., 2011; Antonova et al., 2012). Overall though, our results suggest that OEH is a more potent regulator of egg development in *G. atropalpus*. Sequence analysis indicates that mosquito OEH is a member of the neuroparsin family of insect neuropeptides, which share some homology with mammalian insulin growth factor binding proteins. This interaction was demonstrated for a neuroparsin and ILP from *Locusta*

ILP genes (Marquez et al., 2011; Antonova et al., 2012), we think it likely that all anautogenous species rely on the release of one or both of these neuroendocrine factors to stimulate egg maturation. We further suggest the likely key factor in the evolution of autogeny by *G. atropalpus* and other species is the shift from blood meal-dependent to blood meal-independent regulation of OEH and/or ILP release. How OEH and ILP release is regulated in contrast is unknown in any species. In *A. aegypti*, the medial neurosecretory cells in the female brain are the primary site of OEH and ILP synthesis, while storage and release of these peptides occurs in the

migratoria (Badisco et al., 2008), but its functional significance in this insect is not resolved. The primary gonadotropic activity of OEH in two related mosquito species combined with our lack of knowledge of how OEH and other neuroparsins function led us to examine whether OEH activity depends upon the IR. Our RNAi results strongly indicate that the activity of AaILP3 and endogenous ILPs is fully IR dependent, whereas the activity of OEH is not. Previous receptor-binding studies in *A. aegypti* indicate that ILP3 binds with high affinity to the IR (Brown et al., 2008; Wen et al., 2010). In contrast, our preliminary results indicate that OEH does not bind to either the IR or ILPs, which together with the results of this study suggests OEH activity does not depend on direct interactions with either ILPs or their receptor. *Aedes aegypti* is likely the better model for identifying how OEH interacts with the ovary, fat body and potentially other tissues to regulate egg development. Once a candidate receptor and signaling pathway is identified though, studies with *G. atropalpus* could help advance our understanding of OEH function.

LIST OF ABBREVIATIONS

20E	20-hydroxyecdysone
AaILP	<i>Aedes aegypti</i> ILP
Aar/OEH	<i>A. aegypti</i> recombinant long OEH
ECD	ecdysteroid hormone
ILP	insulin-like peptide
IR	insulin receptor
JH	juvenile hormone
OEH	ovary ecdysteroidogenic hormone
PE	post-eclosion
RIA	radioimmunoassay
RNAi	RNA interference
TOR	target of rapamycin
VG	vitellogenin

ACKNOWLEDGEMENTS

We thank Sarah Robertson, Anne Robertson and Amy Johnson for their assistance in maintaining the mosquito colony.

FUNDING

This work was supported by the National Institutes of Health (NIH) [grant AI33108] to M.R.S. and M.R.B. Deposited in PMC for release after 12 months.

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