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Isolation of an insulin-like peptide from the Asian malaria mosquito, *Anopheles stephensi*, that acts as a steroidogenic gonadotropin across diverse mosquito taxa

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Abstract

Many insulin-like peptides (ILPs) have been identified in insects, yet only a few were isolated in their native form for structural and functional studies. Antiserum produced to ILP3 in *Aedes aegypti* was used in a radioimmunoassay to monitor the purification of an ILP from heads of adult *An. stephensi* and recognized the ILP in other immunoassays. The structure of the purified peptide matched that predicted for the ILP3 in this species. The native form stimulated ecdysteroid production by ovaries isolated from non-blood fed females. Synthetic forms of *An. stephensi* ILP3 and ILP4 similarly activated this process in a dose responsive manner. This function was first established for ILP3 and ILP4 homologs in *Aedes aegypti*, thus suggesting their structural and functional conservation in mosquitoes. We tested the extent of conservation by treating ovaries of *An. gambiae*, *Ae. aegypti*, and *Culex quinquefasciatus* with the *An. stephensi* ILPs, and both the native and synthetic ILP3 were stimulatory, as was the ILP4. Taken together, these results offer the first evidence for ILP functional conservation across the *Anophelinae* and *Culicinae* subfamilies.

Keywords

neuropeptide; neurosecretory cells; ecdysteroid hormone; ovary

1. Introduction

The ever-increasing number of genomic databases for insects has revealed multiple genes encoding insulin-like peptides (ILPs) in each species, thus affirming the broad conservation of this important family of peptide hormones (Antonova et al., 2012; Nässel and Vanden Broeck, 2016). The translated products of ILP genes are pre-propeptides with B, C, and A chains that share structural motifs with insulin, insulin-like growth factors, relaxin, and other related ILPs first identified in mammals and other vertebrates. The first insect ILP (bombyxin) to be characterized was isolated after many chromatographic steps from large-scale extracts of heads from the silkworm, *Bombyx mori*, based on molt and growth

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promoting activity (Ishizaki and Suzuki, 1994; Mizoguchi and Okamoto, 2013). Structural studies determined it to be a heterodimer of peptide chains that bore striking similarity to the A and B chain sequences of insulin, and the positionally conserved cysteine residues formed two disulfide bonds linking the chains and another intra-A chain bond, as for vertebrate insulin. Later, cDNA was cloned from *B. mori* and found to encode the canonical pre-propeptide, indicating a C chain that was cleaved post-translation from the bioactive ILP. Chemically synthesized forms of *B. mori* ILPs also were shown to activate ecdysteroid hormone production by prothoracic glands in silkworms or bind to a putative insulin receptor (Ishizaki and Suzuki, 1994; Büllsbach, 1999; Nagata, 2010; Mizoguchi and Okamoto, 2013; Smith et al., 2014; Gu et al., 2015). Subsequently, this information led to the identification of more than 30 ILP genes in this species (Aslam et al., 2011), but the extent of their functional significance has not been explored. Since then, only two other insect ILPs have been isolated - from the locusts *Locusta migratoria* and *Schistocerca gregaria*, but their function is unknown (Hetru et al., 1991; Badisco et al., 2008).

Among insects, the function and signaling of ILPs are best characterized for two species of Diptera, *Drosophila melanogaster* and the yellow fever mosquito, *Aedes aegypti* (Nässel, 2012; Antonova et al., 2012; Strand et al., 2016; Nässel and Vanden Broeck, 2016). Genes encoding the eight ILPs in these insects were identified only after genomic databases were made available, and ILPs are expressed mostly in tens of cells located in the brain and throughout the nervous system (Brogiolo et al. 2001; Riehle et al., 2006). Evidence for the pluripotency of ILPs in *D. melanogaster* is derived largely from the indirect effects of genetic mutations that affect ILP or signal protein expression, but never with isolated endogenous ILPs (Nässel, 2012; Antonova et al., 2012; Mizoguchi and Okamoto, 2013; Nässel and Vanden Broeck, 2016). Such genetic manipulations have not been achieved with *Ae. aegypti*, so based on the findings that bovine insulin activated ecdysteroid hormone production by ovaries of this species (Graf et al, 1997; Riehle and Brown 1999) and that ILP3 (AaILP3) is the most similar homolog of mammalian insulin, a modified form of the ILP3 was chemically synthesized. It was more potent than bovine insulin in activating ecdysteroid production *in vitro* and egg maturation in blood-fed females and was specifically bound by the insulin receptor expressed in mosquito ovaries (Brown et al., 2008). Other functions and receptor binding of synthetic AaILP3 were characterized later and found to differ from that of a synthetic form of AaILP4 (Wen et al., 2010; Gulia-Nuss et al., 2011; Gulia-Nuss et al., 2011; Gulia-Nuss et al., 2012).

One long pursued goal of this laboratory was to isolate a native mosquito ILP and confirm its bioactivity and structure. After many attempts over the last 20 years, we again took on this task after identifying five ILP genes and profiling their expression in the Indian malaria mosquito, *Anopheles stephensi* (Marquez et al., 2011), and the annotation of this species' genome provided a new resource for characterizing ILP and signaling pathway genes (Jiang et al., 2014). Specific antisera produced to ILPs identified in the African malaria mosquito (Krieger et al., 2006) were used to identify cellular sources in *An. stephensi* (Marquez et al., 2011), and they were evaluated in our preliminary study for use in a radioimmunoassay (RIA) along with antisera to AaILP3 for recognition of ILP-like immunoreactive factor(s) present in crude extracts of *An. stephensi* adult heads. Only the AaILP3 antiserum had a suitable affinity and specificity for the extracted factor, thus it was used in an RIA to monitor the

purification of a native ILP through a few chromatography steps. The isolated peptide activated ecdysteroid production by ovaries of this species, and its mass matched that of the predicted ILP3 homolog in this species (AsILP3; Marquez et al., 2011). Based on this information, the predicted bioactive form of AsILP3 was synthesized to determine if it was similarly bioactive. As well, AsILP4 was synthesized so that it could be tested in the same bioassay along with the isolated and synthetic ILP3 with ovaries from *An. stephensi* and other mosquito species. Our results show that two different ILPs can activate a key step in egg development in representative species spanning a medically important insect family.

2. Materials and methods

2.1 Mosquito rearing

The *An. stephensi* colony was obtained from the laboratory of S. Luckhart (University of California, Davis), which originated from the colony at the Walter Reed Army Institute of Research. Mosquitoes were maintained at 28°C, 70% humidity, and a 16 h light/8 h dark cycle. Larvae were fed a daily regimen of ground TetraMin® fish food (120 larvae per pan/500 ml water) until pupation, and adults had access to 10% sucrose. Colonies of *Aedes aegypti* (UGAL strain), *An. gambiae* (G3 strain), and *Culex quinquefasciatus* (CDC strain) were maintained as above, but larvae were fed a mixture of ground rat chow, lactalbumin, and brewer's yeast as described previously (Riehle et al., 2006).

2.2 ILP3 antiserum production

We produced an antiserum to AaILP3 for use in different immunoassays. As antigens, the predicted A chain (SPRGGIVDESSLRPSSINQLLKYSKTIA) and B chain (ADQRFSGKQLVLTLSMLSDEFDPDLHYGA) were synthesized on an Applied Biosystems 433 synthesizer using standard F-moc chemistry. The peptides were cleaved from the resin, purified by HPLC (peptide structures confirmed by mass spectrometry), and then conjugated to thyroglobulin with glutaraldehyde (Harlow and Lane, 1988). This complex was provided to Pacific Immunology (Ramona CA) for antisera production in rabbits. Preimmune sera and antisera from different bleeds were screened for recognition of synthetic AaILP3 spotted on PVDF membrane and visualized by chemiluminescent detection. The diluted serum (1:1000 and lower) from the final bleed of rabbit 3580 was found to detect submicrograms of the AaILP3, so it was used in the immunoassays described below.

2.3 Immunocytochemistry

With the AaILP3 antiserum, immunostained cells were not well resolved from background fluorescence of tissues processed as below, but after affinity purification (SulfoLink column kit; Thermo Scientific, Waltham, MA), the staining with antibodies was much clearer. Tissues from females (3 to 5 days old) were dissected in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) and fixed for 1–3 h at room temperature, then permeabilized in 4% paraformaldehyde/PBS with 0.2% Triton X-100 for 15 min and washed in PBS. Next, they were treated with goat serum (5%) in PBS with 0.1% Tween 20 (PBS-GS-T) at 4 °C for 1 h, and affinity purified antibody was added (1:100 dilution) for overnight incubation at 4 °C. After rinsing with PBS-GS-T, tissues were incubated in a 1:2000 dilution of goat-anti rabbit Alexa Fluor 488F® (Molecular Probes, Eugene, OR) in

PBS-GS-T overnight. Tissues were washed, slide mounted, and images captured with a digital camera (JVC Americas Corp., model KY-F70BU) on an Olympus® BX60 microscope using Auto-Montage Pro software (Synoptics Ltd., version 5.01.0005). For the preabsorption control, diluted antibody was absorbed with the AaILP3 A and B peptides (10^{-4} M) overnight before use.

2.4 ILP3 radioimmunoassay

A bioactive form of AaILP3 (norLeu substituted for Met) was synthesized in the laboratory (Brown et al., 2008) and later by CPC Scientific, Inc. (Sunnyvale, CA). The synthetic peptides (90% purity) were rehydrated in pure water as a 200 μ M stock, aliquoted, stored at -80°C , and used as described below. AaILP3 was radiolabeled with ^{125}I using the lactoperoxidase method, and the peptide peak with the highest specific activity was purified by HPLC, as monitored with an in-line gamma counter (Crim et al., 2002). A competitive RIA, modified from Nuss et al. (2010), was based on the radiolabeled peptide (15,000 counts per minute (CPM)/tube), AaILP3 antiserum (1:100), and synthetic AaILP3 standards. Aliquots with 500 to 2,000 *An. stephensi* head equivalents were taken from HPLC fractions, lyophilized, and resuspended in RIA buffer (0.05 M Tris-HCl, pH 7.2; 0.1% bovine serum albumin; 0.02% sodium azide). Duplicate samples of the fractions were mixed with antiserum and radiolabeled AaILP3 in glass tubes (5×60 mm) and incubated overnight at 4°C . Unbound peptide was precipitated with a charcoal-dextran mixture by centrifugation, and after removal of the supernatant, pellets were loaded on a Cobra™ II AutoGamma counter (Packard Instrument Company). The amount of immunoreactive peptide in aliquots from extracts or fractions was estimated from a linear regression equation calculated from the bound/free CPM ratios for a range of 0.1 to 5 pmol of synthetic AaILP3.

2.5 Mosquito head extraction and HPLC purification of an ILP

An. stephensi adults (5 days old) were collected into 50 ml tubes and stored at -80°C . The tubes were vigorously shaken to break frozen heads from the bodies, and heads were sifted through fine mesh screens at 4°C for collection. Approximately 84,000 heads (as estimated by weight: 69 μg /head; 5.8 g of heads) were macerated in 30 ml of extraction solution (90% MeOH /1% TFA) with a Super Dispax Tisumizer (Tekmar), followed by probe sonication for 1 min on ice. The extract was frozen (-80°C , 0.5 h), thawed, and centrifuged (10,000 rpm, 15 min, 4°C). After removing the supernatant, the pellet was extracted again, as above, and then stored at 4°C overnight before centrifugation and supernatant removal.

The two supernatants were pooled, dried in a vacuum centrifuge, resuspended in 49 ml of water with sonication, vortexed, centrifuged ($5,000 \times g$, 10 min, 4°C) and supernatant was collected. This process was repeated on the pellet, and resulting supernatants were pooled and transferred to a series of 15 ml conical glass tubes (7 ml in each). Two sequential chloroform extractions were performed by adding 3.5 ml of chloroform to each tube, vortexing, and centrifuging ($5,000 \times g$, 10 min, 4°C). The aqueous layers were pooled in a fresh tube, partially dried in a vacuum centrifuge, lyophilized to dryness, and stored at -80°C . A reddish interphase that formed between the organic and aqueous phases also was collected, lyophilized, and resuspended in water with sonication followed by centrifugation as above. The supernatant was transferred to conical glass tubes, and the pellet was

resuspended and processed as above. Again, the supernatants were pooled, and two steps of lipid extraction were performed as above, using hexane in place of chloroform. Extensive organic extraction of lipids was necessary to minimize their carryover in the aqueous phase that when injected onto a HPLC would irreversibly bind and damage the column and other components. The aqueous phase resulting from this extraction was dried in a vacuum centrifuge and stored at -80°C .

The dried aqueous and interphase extracts were each solubilized with 3 ml of water, and aliquots were taken to quantify the immunoreactive peptide over a dilution series. The remaining extracts were mixed with 10% starting mobile phase, sonicated, and centrifuged ($5,000 \times g$ for 5 min at 4°C). The supernatants were loaded and eluted in the first reversed phase HPLC step on a Beckman 126/166 chromatography unit (Fig. 1). Afterwards, aliquots were taken from the fractions and screened for immunoreactive peptide in the ILP3 RIA. Immunoreactive fractions were pooled, greatly reduced in volume by vacuum centrifugation, and mixed with 10% mobile phase for loading onto the column for the second HPLC step. Two successive steps were needed to isolate an immunoreactive peptide to apparent homogeneity in a single peak (Fig. 1 and Fig. 3).

2.6 Ovary ecdysteroid production bioassay

Immunoreactive peptide in fractions from each HPLC step was tested for activity in the *in vitro* ovary bioassay, adapted from Sieglaff et al. (2005). Briefly, two pairs of ovaries from non-blood fed, virgin *An. stephensi* females were incubated with the resuspended material in Sf-900 II SFM media (Gibco, Life Technologies; 60 μl) for 6 h, 27°C . Ecdysteroids secreted into the media (50 μl) were quantified with a RIA. After the native *An. stephensi* ILP3 was isolated, its dose response activity was compared in the bioassay with ovaries from non-blood fed, virgin *An. stephensi* and *An. gambiae* and from non-blood fed, mated *Ae. aegypti* and *C. quinquefasciatus*, along with synthetic *An. stephensi* ILP3 and ILP4 (CPC Scientific, Inc.), as predicted (Marquez et al., 2011; Fig. 7D). The synthetic peptides (90% purity) were rehydrated in pure water as a 200 μM stock, aliquoted, stored at -80°C , and assayed for bioactivity as above.

2.7 ILP immunoblot procedure

The procedures described below are the outcome of many trials to determine the best practices to separate ILPs by gel electrophoresis and transfer to membranes for chemiluminescent detection with the AaILP3 antibody. The goals were to determine whether the isolated immunoreactive peptide was the expected size of an ILP and the specificity of the antibody. For one procedure, AaILP3 and isolated *An. stephensi* ILP3 (16,000 head equivalents of HPLC fraction 51, lyophilized) were solubilized in 15 μl water and 15 μl sample buffer (125 mM Tris [pH 6.8], 50% glycerol, 4% SDS, 0.02% bromophenol blue), and incubated at 55°C for 30 min. After centrifugation ($5,000 \times g$, 5 min, 4°C), the material was loaded into a lane of a 16.5% Tris-tricine gel (BioRad Mini-Protean) and resolved by electrophoresis (94 V, 180 Ma, 3.5 h, 4°C) along with molecular weight markers (Spectra Multicolor Low Range Protein Standards, Fermentas). Peptides were transferred to PVDF (BioRad, protein sequencing grade, 0.2 μm) for 80 min in a tank with Tris-glycine buffer with 10% MeOH (45 V, 60 Ma, 4°C). After rinsing with water, the blot was dried overnight

and then rewet with 100% MeOH followed by Tris buffered saline with 0.1% Tween 20 (TBS-T), and transferred to TBS-T with blocking agent (0.1 g/10 ml; ECL Advance kit, GE HealthSciences) and 1% goat serum for 2 h at room temperature. The blot was incubated overnight with AaILP3 antiserum (1:1000 dilution) at 4 °C. After washing, blots were treated with goat anti-rabbit IgG-peroxidase conjugate (1:20,000 dilution; Sigma–Aldrich) in fresh blocking solution for 4 h at 25 °C. Blots were washed with TBS-T and developed for chemiluminescent detection (ECL Advance kit, Amersham or Clarity ECL substrate, BioRad) with the GeneGnome imaging system (Syngene, Frederick, MD). Later, this procedure was modified for the detection of synthetic AaILP3, AsILP3, AsILP4, and human insulin (Sigma-Aldrich), along with synthetic AaILP4 and AaILP8 (CPC Scientific, Inc.), as predicted (Riehle et al., 2006; Fig. 7D). The second procedure followed the same procedure for electrophoresis and immunodetection as above, but the peptides in the gel were semi-dry transferred to 0.1 µm nitrocellulose (Whatman, Protran BA 79) in a BioRad Trans-Blot® Turbo™ Transfer System with 20% MeOH Tris-glycine buffer (15 min, minigel program). Membranes were then rinsed, treated with a stripping solution (1 g glycine, 30 µl Tween 20, and 300 µl β mercaptoethanol to 30 ml water), while rocked at 60°C for 1 h, exhaustively rinsed with water, and dried overnight. In earlier trials, no ILP3 bands were observed on blots that were not stripped, but when stripped, bands were evident, suggesting this treatment exposed epitopes for antiserum binding.

3. Results

3.1 Brain neurosecretory cells contain an ILP-like peptide

With whole tissue immunocytochemistry, we found that affinity purified antibodies from the AaILP3 antiserum recognized ILP secreting cells in female *An. stephensi*. A cluster of 8 to 10 medial neurosecretory cells was clearly stained in the brain of adult females (Fig. 2A). Axon tracts extended from these cells through and out of the brain to the corpus cardiacum, a neurohemal organ, and along the anterior midgut (Fig 2C). In addition, a pair of cells in each of the abdominal ganglia was stained, as were their axons extending along the body wall (Fig. 2B). Immunostaining was not observed in the midgut or other tissues of females or in the nervous system of females treated with AaILP3 preabsorbed antibodies (results not shown).

3.2 Native AsILP3 was purified from head extract

As confirmed above, the head of an *An. stephensi* female, which encloses the brain with ILP-containing cells, is an enriched source relative to the rest of the body. Accordingly, adult mosquito heads were harvested for studies to determine optimal conditions for the extraction of heads and which of the different ILP antisera available in the laboratory recognized extracted immunoreactive material in the RIA. The procedure described herein gave the highest yield of ILP-immunoreactive factors best recognized by the AaILP3 antiserum. Mass extraction of more than 80,000 heads in 90% MeOH/1% TFA followed by two steps of organic solvent/water phase separations yielded an estimated 175 pmol of ILP-like peptide in the aqueous phase and 240 pmol in the aqueous-organic interphase. After drying, the two phases were injected separately for the first HPLC step, and the majority of ILP3-like peptide eluted at nearly the same time, so the fractions were pooled for the next HPLC step

(Fig. 1). Three subsequent HPLC steps were needed to further purify the ILP3-like peptide. We also tracked whether the immunoreactive peptide purified at each step stimulated ovary ecdysteroid production by ovaries taken from virgin, non-blood fed *An. stephensi*, and the fact that it did indicated extraction and HPLC had not affected its bioactive structure. Flanking HPLC fractions also were tested and did not activate ovaries.

In the last step, a single peak was resolved and collected in fraction 51 (F 51, Fig. 3) representing the remaining 69,000 head equivalents (hd eq). Two aliquots (10,000 hd eq) were taken from fraction 51 to determine the mass of intact and reduced peptide by matrix-assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOF; University of Georgia Proteomics and Mass Spectrometry Facility, Athens, GA). The observed mass of the isolated peptide (6274.2 Da, Fig. 4 top) most closely matched that predicted for the post-translationally modified AsILP3 with an extended B chain and three disulfide bonds (6273.3 Da, Fig. 5) and not that of other AsILPs (Marquez et al., 2011). AsILP3 A and B chains were predicted to have masses of 3206.8 Da and 2958.4 Da, respectively, assuming cleavage of the C chain and signal peptide (Fig. 5). After reduction of the intact, isolated peptide (1.8 μ M dithiothreitol for 1 h at 90 °C), a mass of 3207.6 Da corresponding to that predicted for the A chain was observed (Fig. 4 bottom). The other predominant mass of 3074.8 Da did not correspond to the mass above, but it was a good fit with 3072.5 Da predicted for the B chain beginning with asparagine at the amino-terminus (Fig. 4 and 5). These results in total indicate that a native form of *An. stephensi* ILP3 was isolated.

3.3 Native AsILP3 stimulates ecdysteroid production by mosquito ovaries

A sufficient amount of native AsILP3 remained to be tested in bioassays with ovaries from different mosquito species to determine whether their dose responsiveness was similar. Preliminary incubations with ovaries from non-blood fed, presumably mated *An. stephensi* and *An. gambiae* females often resulted in high ecdysteroid quantities being detected in the media with the RIA, even in controls containing no ILPs. The ovaries had the last two abdominal segments attached, which may have included the mating plug and spermatheca containing ecdysteroids transferred from males, as first reported for this species (Pondeville et al., 2008) and later for other Anopheline species, including *An. stephensi* (Mitchell et al., 2015). Therefore, ovaries from virgin *An. gambiae* and *An. stephensi* females were used to avoid contamination from male ecdysteroids. *Ae. aegypti* and *Cx. quinquefasciatus* males do not transfer ecdysteroids, so ovaries from females of undetermined mating status were used for the assay. Aliquots of the isolated AsILP3 (F 51) were diluted over a dose range of 0.2 to 200 hd eq for incubation with ovaries from non-blood fed females of the four species. The peptide at 200 hd eq stimulated ecdysteroid production to a significantly greater level than that of control ovaries from each of the four species (Fig. 6). At this dose, ovaries of the two *Anopheles* and *Culex* spp. produced over 200 pg of ecdysteroids over 6 h, but the *Ae. aegypti* ovaries were highly responsive to both 20 and 200 hd eq, producing approximately 400 and 530 pg ecdysteroids, respectively.

3.4 Synthetic AsILP3 stimulates ecdysteroid production by mosquito ovaries

To verify the above results, synthetic AsILP3 and AsILP4 were used to compare dose activation in the bioassay with ovaries from the four species. Both of the synthetic ILPs over the range of 0.2 to 20 pmol stimulated ecdysteroid production to a significantly greater level than that of control ovaries from *An. stephensi* and *An. gambiae* (Fig. 6). Again *Ae. aegypti* ovaries were highly responsive to the AsILP3 even down to a 0.02 pmol dose that significantly stimulated ecdysteroid production, but higher doses of AsILP4 (2 or 20 pmol) were required for a comparable effect. In contrast, *C. quinquefasciatus* ovaries were equally responsive to the 20 pmol dose of AsILP3 and AsILP4, but not to 2 pmol (Fig. 6).

3.5 AaILP3 antiserum detects only ILP3 on immunoblots

Both synthetic AaILP3 and AsILP3, to as little as 0.5 µg, were detected on nitrocellulose immunoblots, but synthetic AaILP4, AaILP8 and human insulin, at much greater amounts, were not (Fig. 7 A, C). The isolated immunoreactive peptide in fraction 51 also was detected on sequencing grade PVDF as a band of approximately 7 kDa (Fig. 7B), which agreed with the MALDI-TOF data for the non-reduced peptide.

4. Discussion

The mining of genomic databases for diverse insects readily yields genes encoding ILPs and other peptide hormones, and subsequent bioinformatic analysis provides predictive structural information about the processed peptides. This information, however, fails to establish what forms exist and are functional in an insect (e.g. 26 predicted ILP genes in the genome of the tobacco hornworm, *Manduca sexta*; Kanost et al., 2016). Few successful efforts to isolate insect ILPs for functional studies have been reported as discussed above, and we had three objectives for this study. The first was to develop a simplified procedure for the extraction and purification of an ILP from adult mosquitoes that could be tracked more easily by its immunoreactivity rather than bioactivity. If successful and a sufficient amount of the ILP was isolated, the second objective was to determine whether its bioactivity matched that of a predicted synthetic form, as this has never been demonstrated for an insect ILP. Again if successful, the third objective was to ascertain the extent to which ILP function was conserved between ILPs and across culicid species.

Antisera produced to *An. gambiae* ILPs (Marquez et al., 2011) and AaILP3 were screened for optimal parameters in the RIA with radiolabeled AaILP3 (the only ILP available at the time) and recognition of immunoreactive material in *An. stephensi* head crude extracts. The AaILP3 antiserum satisfied both criteria, and it detected ILP positive cells in the brains of *An. stephensi* by immunocytochemistry. We had no expectation that only AsILP3 would be isolated from the mass head extract, given the many amino acid motifs shared between AaILP3 and the predicted *An. stephensi* ILPs (Fig. 7D) and unknown differences in ILP abundance and stability during extraction and purification. We also checked that the immunoreactive ILP purified at each chromatography step was bioactive. With these combined methods, a sufficient amount of the pure peptide was obtained to conduct structural and functional studies.

Many previous attempts in our laboratory failed to purify an ILP from mosquitoes, and over time, three problems became evident: 1) low and non-specific binding of ILP antisera in RIAs, 2) low efficiency in extraction and purification, and 3) little to no bioactive material remaining because of repeated bioassays at each of many purification steps. The AaILP3 antiserum was an improvement over previous batches and mitigated the first problem. The next two problems are related and pose the main obstacles. To obtain enough material for extraction, we mass reared nearly 100,000 *An. stephensi* adults and physically separated the heads, which contain brains with the majority of ILP producing cells, thus greatly enriching the ILP content relative to the rest of the biological material to be extracted. The next consideration was that homogenization and solvent extraction of the ILP from the heads must preserve its structure/activity and retain its solubility, while maximizing denaturation or precipitation of extraneous cellular material. Acid-ethanol extraction is commonly applied to the extraction of mammalian insulin from serum and pancreas, and was used successfully for the extraction of insect ILPs (Duve and Thorpe, 1979; Badisco et al., 2008; Maniere et al., 2009). This solvent extraction did not yield detectable ILP in our initial attempt, and a similar failure was noted for *Apis mellifera* (Moreau et al., 1981). Some partial successes have been achieved with saline (Moreau et al., 1981; Maniere et al 2000, 2004), and the first extraction of an ILP from locusts was achieved with a 1 M acetic acid solution (Hetru et al 1991). We found that a 90% MeOH/0.1% TFA solution, used to extract blowfly heads for peptides that stimulated ecdysteroid production by ovaries (Maniere et al., 2009), gave the best yield of ILP-like material from mosquito heads. During the extraction process, we tracked the recovery of ILP immunoreactivity at each step and were surprised that the hydrated material from the pooled supernatants and the interphase between the chloroform with lipids, contained nearly equal amounts of ILP-like material. By including both in the next step, we doubled our yield. This finding underscores the complexity of extraction and suggests different solvents used to homogenize insect tissues should be tested for relative preservation and retention of ILPs. Also nonspecific antibody binding to the material in crude extracts can complicate this analysis, but can usually be detected with an extensive dilution of the hydrated extract solution to where the ILP values are in accord with the dilution steps.

The antiserum produced to AaILP3 was the key to successful isolation of the AsILP3, and it stained specific cells in the nervous system of *An. stephensi* females. The expected source of AsILP3 isolated in this study ILP are the stained cells observed in brain. Their axons exited the brain and extended to the neurohemal organ and along the anterior midgut of females. These cells appear to be the same ones identified in a previous immunocytochemical study of *An. stephensi* and *An. gambiae* females with an antibody produced to a composite peptide representing the A chains of ILP1, 3, and 4 in *An. gambiae* (Marquez et al., 2011). Similarly positioned cells were stained in the brains of *Ae. aegypti* and *An. gambiae* females with antisera to the A chain of *Drosophila* ILP2 (Cao and Brown, 2001) and to a silkworm ILP (bombyxin II; Krieger et al., 2004; Riehle et al., 2006; Brown et al., 2008). In total, these results suggest that ILP3 homologs are expressed primarily in the brain medial neurosecretory cells across aedine and anopheline mosquitoes. Whether or not other ILPs are produced in these same cells is uncertain, and no other immunoreactive material in the

different chromatographic steps was detected with the RIA, suggesting the antiserum was specific for conserved ILP3 epitopes.

To test this hypothesis, we developed immunoblot procedures for the detection of ILPs to determine whether the isolated immunoreactive peptide was structurally similar to AaILP3 (Fig. 7). Other synthetic mosquito ILPs and human insulin were similarly treated and blotted but not detected, thus affirming the antiserum specificity for ILP3-like peptides. Earlier attempts to detect ILPs after electrophoresis and blotting failed, so the same procedure was carried out with radiolabelled AaILP3, and the different solutions used to transfer or wash the gel and membrane were checked for radioactivity. We found that most of the transferred peptide did not remain attached to the membrane during the washing and antibody treatment steps. To retain more peptide, we used sequencing grade PVDF to which most of the isolated peptide in fraction 51 was transferred and detected (Fig. 7B), as well as AaILP3 at 5 µg was included and detected (not shown). From our calculations above, the isolated peptide band represented approximately 16,000 hd eq for visualization, and further attempts to quantify the peptide by immunoblot would have quickly exhausted our starting material during purification. We then found that by transferring smaller amounts of synthetic AaILP3 and AsILP3 to nitrocellulose with a smaller pore size and stripping the blot, as little as 0.5 µg of ILP3 could be detected. As discussed above and shown for the RIA, a low concentration (1:100) of the ILP3 antiserum is required even in the sensitive RIA, which can detect approximately 0.4 to 40 ng in solution. We have encountered the same problem in other attempts to produce antisera to exogenous ILP antigens, and are uncertain as to why such antigens may have low immunogenicity in rabbits. Whether or not the immunoblot technique or RIA can be made more sensitive for the detection and quantification of physiological amounts of ILP3 in relatively few mosquitoes remains to be determined.

Subsequent mass spectrophotometry analysis confirmed that masses obtained from both the intact and reduced forms of the isolated immunoreactive peptide matched those predicted for AsILP3. Given the limited amount of native AsILP3 isolated and its demonstrated bioactivity in the ovary ecdysteroid production bioassay, we had it and the predicted AsILP4 synthesized to compare bioactivity. The amount of ecdysteroids produced by *An. stephensi* ovaries was similar for 0.2 pmol of synthetic AsILP3 and 20 hd eq of native AsILP3. Assuming that the synthetic AsILP3 retains the same bioactivity as the native molecule, this provides an estimate of the final amount of native AsILP3 isolated as approximately 690 pmol (~4.3 µg) from the final extract of *An. stephensi* heads or approximately 62.4 pg/hd eq. This total is more than the approximately 400 pmol estimated for the original extract with the RIA and indicates little was lost, and more importantly bioactivity was retained during the purification steps.

Clearly, this extraction and purification procedure in combination with the RIA yielded a reasonable amount of the AsILP3, but it would have been insufficient for additional studies. Synthesis of ILPs is a technical challenge in that specific cysteines must form disulfide bonds essential for proper folding and bioactivity. The previous in-house effort to synthesize AaILP3 was long and complicated but finally successful (Brown et al., 2008) and enabled the elucidation of its binding to the insulin receptor and diverse functions in *Ae. aegypti* (Wen et al., 2010; Gulia-Nuss et al., 2011; Castillo et al., 2011; Gulia-Nuss et al., 2012;

Gulia-Nuss et al., 2015; McKinney et al., 2016). Fortunately, commercial peptide synthesis has advanced greatly and synthesis of AsILP3 and AsILP4 in milligram quantities was a reasonable expense, given the bioactivity and stability of the product.

An earlier study showed that synthetic AaILP3 and AaILP4 stimulated ecdysteroid production by ovaries of *Ae. aegypti* in a persistent but differentially dose responsive manner (Wen et al., 2010), yet it was not known whether this stimulatory function of ILPs was conserved across mosquito taxa. With the availability of the native AsILP3 and synthetic forms of AsILP3 and AsILP4, we tested the relative dose responsiveness of ovaries from *Anopheles*, *Culex*, and *Aedes* species, which represent an evolutionary separation of an estimated 220 million years from the basal *Anopheles* to the *Aedes* (Reidenbach et al. 2009). We did not expect the ovaries of all species to be near-equally responsive to the highest doses of the native and synthetic AsILP3 and even to AsILP4, but they were (Fig. 6). Most surprising was the high sensitivity of *Ae. aegypti* ovaries to the synthetic AsILP3 relative to the other species in that as little as 0.02 pmol significantly stimulated production relative to control ovaries, and higher doses elicited the production of almost two fold more ecdysteroids (500–600 pg) than that of ovaries from the other three species (200–400 pg) during the incubation period. Overall, the *Culex* ovaries were least responsive to the ILPs, and the *Anopheles* ovaries were equally dose responsive to both AsILP3 and AsILP4. For *Ae. aegypti* ovaries, approximately two fold more AaILP4 than AaILP3 was required to stimulate the same level of ecdysteroid production *in vitro* (Wen et al., 2010). This may reflect a difference in signaling as AaILP3 was shown to competitively bind to the insulin receptor, but not AaILP4, which bound to a much smaller receptor protein in ovary extracts (Wen et al., 2010). Also, AaILP3 acts as an “insulin” in sugar fed females and stimulates hemocyte proliferation and trypsin activity in blood fed females (Brown et al., 2008; Castillo et al., 2011; Gulia-Nuss et al., 2011). At this time, it is not known to what extent the cellular expression, signaling, or these pleiotropic functions are unique to ILP3 homologs or shared with ILP4 and other ILPs identified in *Ae. aegypti* and the two *Anopheles* species. For now, these results offer the first demonstration of the conserved gonadotropic function of ILP3 and ILP4 across an important insect group, the Culicidae.

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Highlights

- A native bioactive ILP3 was purified from heads of adult *An. stephensi* mosquitoes.
- Synthetic AsILP3 and 4 stimulated ecdysteroid production by ovaries of this species.
- Ovaries from *Culex* and *Aedes* species were similarly stimulated by the ILPs.

HPLC	ILP3 immunoreactive fractions pooled for next step		Column/ Solvent B	Solvent Gradient/ Flow Rate
Step 1	Aqueous extract F. 53-54	Aqueous-organic interphase extract F. 52-53	Phenomenex Jupiter C18 Semi-prep column 300Å; 250 x 10 mm 80% CH ₃ CN / 0.1% HFBA	10 to 70% B 60 min; 2 ml / min
Step 2	F. 57-60		BioRad RP318 C18 300Å 250 x 4.6 mm 80% CH ₃ CN / 0.1% HFBA	25 to 50% B 50 min; 1 ml / min
Step 3	F. 58-59		BioRad RP318 250 x 4.6 mm 80% CH ₃ CN / 0.1% TFA	29 to 44% B 60 min; 1 ml / min
Step 4	F. 51		Phenomenex Jupiter C5 300Å; 250 x 4.6 mm 80% CH ₃ CN / 0.1% TFA	33 to 45% B 60 min; 1 ml / min

Figure 1.
Sequence of HPLC steps for the purification of native AsILP3 from the two aqueous cuts of the extract of *An. stephensi* adult heads. HPLC fractions with ILP3 immunoreactivity were pooled for each next step and loaded onto the column and subjected to the elution conditions given. Heptafluorobutyric acid (HFBA) or trifluoroacetic acid (TFA) were used as ion pairing agents in the mobile phases, and elution was monitored at 275 or 206 nm, respectively.

Figure 2.

Immunocytochemistry with AaLP3 antibody revealed cells in the nervous system of *An. stephensi* adult females. A. Brain with stained medial neurosecretory cells (arrowhead). B. Ventral nerve cord ganglion (dotted gray outline) with stained cell bodies (arrowhead) and axon processes extending along body wall (arrows). C Anterior midgut with axon processes extending from the MNCs of the brain (arrows). Autofluorescence of sphere-like midgut contents is evident. Scale bars = 100 μ m.

Figure 3.

Elution of the native AsILP3 as a single peak in HPLC step 4 (top) and corresponding fractions (bottom) with the amount of immunoreactive ILP3-like peptide (gray bars) in an aliquot of 2,000 *An. stephensi* head equivalents, as estimated by radioimmunoassay.

Figure 4. MALDI-TOF mass spectroscopy readout of native AsILP3 showing the mass of the non-reduced form (top) and resultant B and A chains of the reduced forms (bottom).

Figure 6.

Native (F 51), synthetic AsILP3 (ILP3), and AsILP4 (ILP4) stimulated ecdysteroid production by ovaries of non-blood fed females representing the three medically important taxa: *Anopheles*, *Culex*, and *Aedes*. Ovaries (two pairs in 60 µl of media) were incubated with doses of native AsILP3 (represented as head equivalents/60 µl) or synthetic AsILP3 and AsILP4 (pmol/60 µl) for 6 h, 27 °C. Secreted ecdysteroids in the collected media were quantified with the ecdysteroid RIA. Bars represent mean ecdysteroid pg ± standard error (SE) for triplicate samples for each of three experiments with different female cohorts for each species (N = 9). Control ovaries were incubated in media only. Results represent significant differences between control and treatments (Dunnett's multiple comparisons test, *P < 0.05; **P < 0.001; ***P < 0.0001).

Figure 7.

Immunoblot detection of native and synthetic AsILP3 and AaILP3. **A.** Representative blot of synthetic AaILP3 and AsILP3 (0.25–2 μ g) separated in a Tris-tricine gel, transferred to nitrocellulose, treated to reducing conditions, and subjected to chemiluminescent detection. **B.** Blot of native AsILP3 (F51) detected after treatment of an aliquot (~16,000 head equivalents) as above but transferred to PVDF and not subjected to reduction treatment. **C.** Representative blot of synthetic AaILP3 (2.5 μ g), AaILP4 (5 μ g), AaILP8 (5 μ g), and AsILP4 (4 μ g), along with human insulin (hIns, 0.6–10 μ g) processed and detected as described in **A**. Molecular weight markers in kilodaltons (kDa) are indicated on left side of blots shown in **A** and **C**. **D.** Amino acid alignment of *An. stephensi* and *Ae. aegypti* ILPs and human insulin used in blots **A** and **C** to highlight amino acid regions identical to the AaILP3 antigen. AsILP3 is most similar and the others are less so, which may explain the lack of antiserum recognition on immunoblots.