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Identification and characterization of a sex peptide receptor-like transcript from the western tarnished plant bug *Lygus hesperus*

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Abstract

Lygus hesperus **females exhibit a post-mating behavioural switch that triggers increased egg laying and decreased sexual interest. In *Drosophila melanogaster*, these changes are controlled by sex peptide (SP) and the sex peptide receptor (DmSPR). In *Helicoverpa armigera*, SPR (HaSPR) also regulates some post-mating behaviour; however, myoinhibiting peptides (MIPs) have been identified as the SPR ancestral ligand, indicating that SPR is a pleiotropic receptor. In the present study, we identified a transcript, designated *L. hesperus* SPR (LhSPR), that is homologous to known SPRs and which is expressed throughout development and in most adult tissues. LhSPR was most abundant in female seminal depositories and heads as well as the hindgut/midgut of both sexes. *In vitro* analyses revealed that fluorescent chimeras of LhSPR, DmSPR and HaSPR localized to the cell surface of cultured insect cells, but only DmSPR and HaSPR bound carboxytetramethylrhodamine-labelled analogues of $DmSP_{21–36}$ and DmMIP4. Injected $DmSP_{21–36}$ also failed to have an effect on *L. hesperus* mating receptivity. Potential divergence in the LhSPR binding pocket may be linked to receptor-ligand co-evolution as 9 of 13 MIPs encoded by a putative *L. hesperus* MIP precursor exhibit an atypical W-X_7-Wamide motif vs the W-X_6-Wamide and W-X_8-Wamide motifs of Drosophila MIPs and SP.**

Keywords: ***Lygus hesperus*****, western tarnished plant bug, sex peptide, sex peptide receptor, MIP, fluorescent microscopy.**

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Introduction

For many insects, accessory gland-derived products transferred in the male's seminal fluid induce changes in the reproductive behaviour of the mated female. In contrast to virgin females, mated females have higher egg-laying rates and are less receptive to male advances. Accessory gland-derived products and other seminal fluid proteins have been linked to significant changes in female gene expression, increases in egg laying, modifications of flight and feeding behaviours, capacity for sperm maintenance and remodelling of the female reproductive tract (Leopold, 1976; Wolfner, 1997, 2002; Gillott, 2003; Avila *et al.*, 2011). While more nuanced understandings of the mechanisms driving insect reproduction can offer insights into the life histories and population dynamics of a given species, targeted disruption of those mechanisms represents intriguing possibilities for novel control strategies. As a consequence, numerous studies have focused on identifying seminal fluid components. One of the most extensively characterized components is the *Drosophila melanogaster* sex peptide (SP), DmSP, a 36-amino acid peptide produced in the male accessory gland that triggers a diverse set of post-mating responses, including juvenile hormone synthesis, perturbed feeding, altered sleep patterns, mobilization of stored sperm, enhanced egg laying, reduced female receptivity and transcriptional regulation of >50 genes (Chen *et al.*, 1988; Aigaki *et al.*, 1991; Moshitzky *et al.*, 1996; Chapman *et al.*, 2003; Liu & Kubli, 2003; Carvalho *et al.*, 2006; Isaac *et al.*, 2010; Gioti *et al.*, 2012). Although it is clear that SP-like factors/activity are present in many species (Leopold, 1976; Gillott, 2003; Avila *et al.*, 2011), the rapid evolution of accessory gland proteins (Swanson *et al.*, 2001; Swanson & Vacquier, 2002; Haerty *et al.*, 2007) has hindered identification of the SP gene outside of *Drosophila*. A genome screen of *Anopheles gambiae*, however, identified a putative SP orthologue (AGAP009352) predominantly expressed in male accessory glands (Dottorini *et al.*, 2007). The product of that gene, though, has poor sequence identity (27%) with DmSP and lacks the tryptophan-rich carboxyl terminus necessary for activity (Kim *et al.*, 2010; Poels *et al.*, 2010).

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Sequence-based screens in the crickets *Gryllus firmus* and *Gryllus pennsylvanicus* (Andrés *et al*., 2006), the honeybee *Apis mellifera* (Collins *et al*., 2006), the Mediterranean fruit fly *Ceratitis capitata* (Davies & Chapman, 2006; Scolari *et al*., 2012), and the tick, *Dermacentor variabilis* (Sonenshine *et al*., 2011), failed to yield an SP orthologue. In the moth *Helicoverpa armigera*, synthetic DmSP stimulates juvenile hormone synthesis in isolated corpora allata (Fan *et al*., 1999) and terminates sex pheromone production both *in vitro* and *in vivo* (Fan *et al*., 2000). Similar results were reported after injections of homogenized *H. armigera* accessory glands (Nagalakshmi *et al*., 2004), which also trigger enhanced egg laying (Jin & Gong, 2001) and are immunoreactive to a DmSP antibody (Nagalakshmi *et al*., 2004, 2007).

Transduction of an extracellular peptide signal such as SP into a biological response frequently requires the involvement of an intermediary cell surface receptor. In recent years, SP has been shown to activate two different *D. melanogaster* G protein-coupled receptors (GPCRs), Methuselah (Ja *et al*., 2009) and a receptor termed sex peptide receptor (SPR) (Yapici *et al*., 2008). Methuselah, a GPCR associated with longevity, mobilized Ca^{2+} in a mammalian cell line in response to 10 μM DmSP, but appears to have no role in mediating mating behaviour in *D. melanogaster* (Ja *et al*., 2009). In contrast, the evidence supporting SPR involvement in mediating the post-mating behaviours of *D. melanogaster* are more convincing – SPR knockdown results in mated females that exhibit virgin behaviours (Yapici *et al*., 2008) and the DmSPR expression profile (female reproductive organs and central nervous system) is consistent with that previously reported for labelled-SP binding (Ottiger *et al*., 2000). Despite little genomic/transcriptomic evidence for SP orthologues, structurally and functionally conserved SPR homologues are present in most insects (Yapici *et al*., 2008; Hanin *et al*., 2011; Šimo *et al*., 2013). The SPR gene from *Aedes aegypti* and *Bombyx mori* mobilized extracellular Ca^{2+} in mammalian cells in response to DmSP (Yapici *et al*., 2008). Furthermore, RNA interference-mediated knockdown of the *H. armigera* SPR (HaSPR) inhibited DmSP-induced suppression of sex pheromone production and reduced the egg-laying response of mated females to that of virgin females (Hanin *et al*., 2012). SPR, however, was unexpectedly shown to be a promiscuous receptor that is also activated by myoinhibiting peptides (MIPs)/allatostatin/prothoracicostatic peptides (Kim *et al*., 2010; Poels *et al*., 2010; Yamanaka *et al*., 2010; Vandersmissen *et al*., 2013). MIPs inhibit spontaneous muscle contractions of insect guts (Schoofs *et al*., 1991; Fónagy *et al*., 1992; Predel *et al*., 2001) as well as inhibit juvenile hormone production in crickets [referred to as allatostatin-B in that system (Stay *et al*., 1995; Bendena *et al*., 1999)] and suppress ecdysteroid biosynthesis in *B. mori* prothoracic glands [termed prothoracicostatic peptides (Hua *et al*., 1999; Liu *et al*., 2004)] but have no effect on female post-mating behaviour (Kim *et al*., 2010; Poels *et al*., 2010). The only shared structural feature between DmSP and DmMIPs is the presence of two carboxyl terminal tryptophan residues (SP: W-X_8-Wamide; MIP: W-X_6-Wamide), which are crucial for SPR activation (Kim *et al*., 2010; Poels *et al*., 2010; Vandersmissen *et al*., 2013). The sexual conflict between males and females that drives the evolution of accessory gland proteins probably also contributed to SPR promiscuity with DmSP co-opting the ancestral MIP receptor (MIPR) to mediate post-mating responses. In tissues/life stages in which SP is absent, however, the receptor retains MIP functionality (Poels *et al*., 2010). This does not preclude the possibility that other species have co-opted SPR in a similar manner. Indeed, the role of SPR in regulating post-mating sex pheromone production in *H. armigera* (Hanin *et al*., 2012) and the presence of a non-SP, non-MIP factor in *Ae. aegypti* that activates SPR (Kim *et al*., 2010) suggests similar evolutionary routes may have been chosen.

In the western tarnished plant bug, *Lygus hesperus* Knight (Heteroptera:Miridae), a polyphagous pest of a wide variety of agronomic and horticultural crops (Scott, 1977; Wheeler, 2001; Goodell, 2009), mating induces changes in both female sexual receptivity (Strong *et al*., 1970; Brent, 2010a, 2010b) and egg-laying (Brent, 2010b; Brent *et al*., 2011). These post-mating changes appear to be linked to seminal fluid transfer, as injection of homogenized spermatophores directly into the hemocoel of virgin females negatively impacted their receptivity to further mating (Brent, 2010a). Given these SP-like mating effects and the structural/functional conservation of SPR in other insects, we hypothesized that reproductive behaviour may be regulated by a similar ligand-receptor system. To assess the validity of this hypothesis, we used homology-based cloning methods to identify a transcript, which we have designated *L. hesperus* SPR (LhSPR), with significant homology to known SPRs. The expression profile of this gene is consistent with a pleiotropic receptor mediating multiple biological functions. A significant difference in expression levels between virgin and mated females is suggestive of a reproductive role. Despite significant sequence conservation with other SPR/MIPRs, heterologously expressed LhSPR was unable to bind fluorescently labelled analogues of DmSP or DmMIP4, both of which bound to DmSPR and HaSPR, suggesting that the LhSPR binding pocket may have diverged. This structural variation in ligand binding may be linked to receptor-ligand co-evolution. A majority (nine of 13) of the MIP-like peptides encoded by the putative LhMIP precursor exhibit a W-X_7-Wamide motif that differs from both DmSP (W-X_8-Wamide) and the MIPs of most holometabolous insects (W-X_6-Wamide).

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Results

LhSPR cloning and sequence characterization

To gain a better understanding of the molecular mechanisms driving the post-mating behaviour switch in Lygus bugs, we sought to clone and characterize the *L. hesperus* SPR (LhSPR). Using degenerate primers designed to conserved amino acid stretches in known SPRs (*D. melanogaster*, ACC68840; *Ae. aegypti*, ABW86944; *B. mori*, NP_001108346; and *Tribolium castaneum*, EFA01285) in conjunction with cDNAs prepared from *L. hesperus* ovary total RNAs, we amplified a fragment of the expected size [120 base pair (bp)]. BLASTX sequence analysis indicated that this fragment was 71% identical to DmSPR. Gene-specific primers designed to this fragment were used in rapid amplification of cDNA ends (RACE)-based PCR to amplify a 490-bp 5′product with 63% sequence identity to DmSPR and an 836-bp 3′ product with 61% sequence identity to DmSPR. Assembly of the consensus RACE products and the degenerate PCR-derived fragment yielded a 1302-bp transcript with a 64-bp 5′ untranslated region (UTR) and a 38-bp 3′UTR containing a poly-A tail (Fig. 1). Multiple independent reactions using cDNA with gene-specific primers designed to the putative start and stop codons generated an amplimer of the expected size (1200 bp). The consensus sequence is predicted to encode a 399-amino acid protein with highest sequence identity (75%) to a pea aphid (*Acyrthosiphon pisum*) sequence annotated as a predicted FMRFamide receptor-like protein. Significant sequence identity was also seen with BmSPR (67% identity, $1e^{-170}$), HaSPR (68% identity, $5e^{-170}$), and DmSPR (62% identity, $3e^{-140}$). Based on this sequence conservation, we have designated the Lh sequence as LhSPR (GenBank accession AEK80439).

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ACATGGGGATATTTTATTCATCGAGTCGGAACCTATCACGTTCTAAGTTCATTGAATTCTCAATATGGTTGAGTTTGAGGACCCTTACTTCAT 93
                                                                  M  V  E  F  E  D  P  Y  F  I 10

CACTAACCTCTCCGATTCCAACCTGGGGGCCAATGAGACGTGGAACTCCACGATCAATCTGGGTGAGGGTGAGCGTCTCGTCAATGTCACCTC 187
 T  N  L  S  D  S  N  L  G  A  N  E  T  W  N  S  T  I  N  L  G  E  G  E  R  L  V  N  V  T  S 41

TGAGATGGATATCGCCTATGCCATACCTCTGTACGGCTACTGCATGCCCTTTCTTCTAGTCATCACGATAGTCGCCAACACGTTGATCGTGGT 279
 E  M  D  I  A  Y  A  I  P  L  Y  G  Y  C  M  P  F  L  L  V  I  T  I  V  A  N  T  L  I  V  V 72

CGTCCTCTCCAAGCGACACATGAGAACTCCGACGAACGCTGTGCTCATGGCGATGGCTCTCTCCGACATGTTCACCTTGCTTTTTCCGGCTCC 372
 V  L  S  K  R  H  M  R  T  P  T  N  A  V  L  M  A  M  A  L  S  D  M  F  T  L  L  F  P  A  P 103
                        deg F
TTGGTTGTTCTACATGTACACCTTCGGAAACCACTATAAACCCCTGTCACCTGTGGGAGCCTGCTATGCATGGAGAGCCATGAATGACATCAT 465
 W  L  F  Y  M  Y  T  F  G  N  H  Y  K  P  L  S  P  V  G  A  C  Y  A  W  R  A  M  N  D  I  I 134
          RACE F/R                                                   deg R
ACCTGGTCTATTCCATACAGCCTCGATATGGCTGACATTGGCACTGGCGGTCCAAAGGTATATCTATGTCTGCCATGCTCCAATGGCGCGTAC 558
 P  G  L  F  H  T  A  S  I  W  L  T  L  A  L  A  V  Q  R  Y  I  Y  V  C  H  A  P  M  A  R  T 165

ATGGTGTACGATGCCCAACGTTCTGAAAGGTGTGGTGTTCATATCCATTTGTGCGACTCTCCACCAGTCACCCAGATTCTTCGAATTAGTGTA 651
 W  C  T  M  P  N  V  L  K  G  V  V  F  I  S  I  C  A  T  L  H  Q  S  P  R  F  F  E  L  V  Y 196

CGAACCCACGACGATAAGATGGGGTGATCAAAACAACGTCGTAGTTTGTAAGGTTAAGTTGGCGGATTGGATAGAGCATGGTTTAACYCCAGA 744
 E  P  T  T  I  R  W  G  D  Q  N  N  V  V  V  C  K  V  K  L  A  D  W  I  E  H  G  L  T  P  D 227

CGTGTACTACACAATCTACTTCCTGTTCAGGGTAATTTTCGTCCACGCCCTTCCTTGCATATCCCTAGTTGCTTTGAACCTCTTGCTGTTCAA 837
 V  Y  Y  T  I  Y  F  L  F  R  V  I  F  V  H  A  L  P  C  I  S  L  V  A  L  N  L  L  L  F  K 258

AGCACTTAGAAAAGCACAAATAAAGAGGAACAAATTGTTCAAAGAGAATAGAAAAGGAGATTGCAAGAAGCTGCGGGACTCCAACTGCACAAC 930
 A  L  R  K  A  Q  I  K  R  N  K  L  F  K  E  N  R  K  G  D  C  K  K  L  R  D  S  N  C  T  T 289

CCTTATGCTCATTGTCGTGGTCACCGTATTCCTRTTCACGGAAATTCCTCTAGCTGTAGTTACGGTGTTGCACATCATATCGAGTAGCATCAA 1023
 L  M  L  I  V  V  V  T  V  F  L  F  T  E  I  P  L  A  V  V  T  V  L  H  I  I  S  S  S  I  K 320

GGAAATCCTCGATTATCACGTGGCCAACATCCTTATCCTCTTCACAAATTTCTTCATCATCTTGAGCTACCCAATCAACTTCGCTATCTACTG 1116
 E  I  L  D  Y  H  V  A  N  I  L  I  L  F  T  N  F  F  I  I  L  S  Y  P  I  N  F  A  I  Y  C 351

TGGGATGTCCCGTCAGTTCCGAGAGACGTTCAAAGAGCTGTTCATAAGAGGTGCTGTACAGGCGACGAGAGGCCGAAATGGAGCCGGAAGCTC 1209
 G  M  S  R  Q  F  R  E  T  F  K  E  L  F  I  R  G  A  V  Q  A  T  R  G  R  N  G  A  G  S  S 382

CAAGTACTCCTTGGTGAATGGCCCGAGGACCAGTACAAACGAGACTGTTCTGTAAGGTTCCTGGTAAAAAAAAAAAAAAAAAAAAAAAAAAA 1302
 K  Y  S  L  V  N  G  P  R  T  S  T  N  E  T  V  L  *                                          399
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Figure 1. cDNA and deduced amino-acid sequence of the *Lygus hesperus* sex peptide receptor (LhSPR). Amino acids are numbered starting at the first in-frame Met (boxed). The first in-frame stop codon is indicated by an asterisk (*). Location of the degenerate and rapid amplification of cDNA ends primers is indicated.

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Table 1. Predicted topology and position of transmembrane domains in LhSPR

		Amino acid residues comprising the predicted helical TM domains						
Algorithm	Topology	TM1	TM2	TM3	TM4	TM5	TM6	TM7
HMMTOP[1]	N-Out, C-In	51–75	88–112	133–151	166–185	232–256	287–311	330–354
Phobius[2]	N-Out, C-In	51–74	86–109	137–156	168–186	234–257	290–315	327–352
Rhythm[3]	N-Out, C-In	51–75	88–112	133–151	166–185	232–256	287–311	330–354
TMHMM[4]	N-Out, C-In	52–74	86–108	135–157	170–187	235–257	290–312	327–349
TOPCONS[5]	N-Out, C-In	52–72	84–104	135–155	170–190	235–255	285–305	331–351
TMPred[6]	N-Out, C-In	51–75	85–106	133–151	172–192	232–254	286–308	327–353
TopPredII[7]	N-Out, C-In	55–75	93–113	132–152	165–185	238–258	285–305	326–346
Consensus	N-Out, C-In	52–74	87–109	134–153	168–187	234–256	287–310	328–351

TM, transmembrane. [1]Tusnády and Simon (2001); [2]Käll *et al.* (2007); [3]Rose *et al.* (2009); [4]Krogh *et al.* (2001); [5]Bernsel *et al.* (2009); [6]Hofmann & Stoffel (1993); [7]von Heijne (1992).

Further analysis of the LhSPR sequence using various algorithms predicted the seven transmembrane-spanning domains, extracellular amino terminus, and intracellular carboxyl terminus that are all characteristic of GPCRs (Table 1).

The predicted LhSPR sequence was aligned with SPR/MIPR sequences from a number of other arthropods (Fig. 2). As expected, highest sequence conservation was observed in the predicted transmembrane domains. Consistent with class A GPCRs (i.e. rhodopsin-like), there is less conservation in the amino terminus and extracellular loop 2 (ECL2), pairwise identity of which drops from 60.5% for the full-length sequences to 38.1%. This drop in sequence conservation is specific to ECL2, as ECL1 and ECL3 (pairwise identity of 61.9% and 55.4% respectively) are relatively well conserved across the seven species examined. *N*-linked glycosylation of GPCR extracellular domains can affect cell surface trafficking and receptor functionality (Duvernay *et al.*, 2005; Wheatley *et al.*, 2012). Scanning the LhSPR sequence for the canonical Asn-X-Ser/Thr glycosylation motif (Kornfeld & Kornfeld, 1985) revealed four potential sites in the amino terminus (Asn12, Asn21, Asn25 and Asn38). Multiple amino terminal *N*-glycosylation sites are also present in the other SPR/MIPR sequences examined; however, only one site (LhSPR Asn38) is conserved across the seven species (Fig. 2), suggesting a potential functional role. All the sequences examined also have conserved Cys residues in ECL2 and ECL1 (Fig. 2) that probably form a disulphide bond that acts to constrain the SPR/MIPR conformation (Wheatley *et al.*, 2012). Class A GPCRs are frequently characterized by a Glu/Asp-Arg-Tyr (E/DRY) motif, located at the boundary between TM3 and intracellular loop 2, that is critical for regulating the inactive/active GPCR conformational states (Rovati *et al.*, 2007). Surprisingly, this motif is mutated in the SPR/MIPR family of GPCRs to Gln-Arg-Tyr (QRY), suggesting that the receptor is likely to adopt an active conformation leading to increased constitutive activity as was reported for DmSPR in mammalian HEK cells (Poels *et al.*, 2010). GPCR phosphorylation is a determining factor in receptor desensitization (Kristiansen, 2004). A scan of the LhSPR sequence revealed multiple potential phosphorylation sites (5 Ser, 1 Thr, and 1 Tyr), most of which are located in the carboxyl terminus (Fig. 2). All but one (Ser393) of these sites are well conserved across the SPR/MIPR sequences examined, suggesting potential roles in receptor regulation.

To more accurately assess the relationship amongst the SPR/MIPR family of GPCRs, we performed a neighbour-joining analysis using LhSPR and 33 additional sequences (26 arthropods, two annelids, two molluscs and one crustacean) identified in a BLASTX analysis of the LhSPR sequence. The respective sequences clustered within well-supported (1000 bootstrap iterations) taxonomic clades (Fig. 3) with LhSPR grouping amongst sequences derived from the hemipteran lineage (i.e. lice, aphids, bugs). Despite varied biological effects, the arthropod SPR and MIPR sequences had the same phylogenetic distribution with no unique clades, suggesting a common ancestral ligand, as has been proposed (Kim *et al.*, 2010; Poels *et al.*, 2010).

Expression profiling of LhSPR

To provide insights into the potential biological functions mediated by LhSPR, we performed endpoint PCR using templates derived from multiple *L. hesperus* developmental stages (from egg to fifth instar) as well as early and late ages of adults. Sequence-specific primers were designed to amplify either a 495-bp fragment of LhSPR or a 555-bp fragment of *L. hesperus* actin, a positive control for constitutive and ubiquitous expression. The LhSPR transcript was amplified from all stages of development examined with eggs exhibiting the most robust amplification (Fig. 4A). LhSPR was likewise amplified with no discernible difference in expression levels from either early (1-day-old) or late (17-day-old) adult virgin *L. hesperus* males and females (Fig. 4A). Similar profiling was

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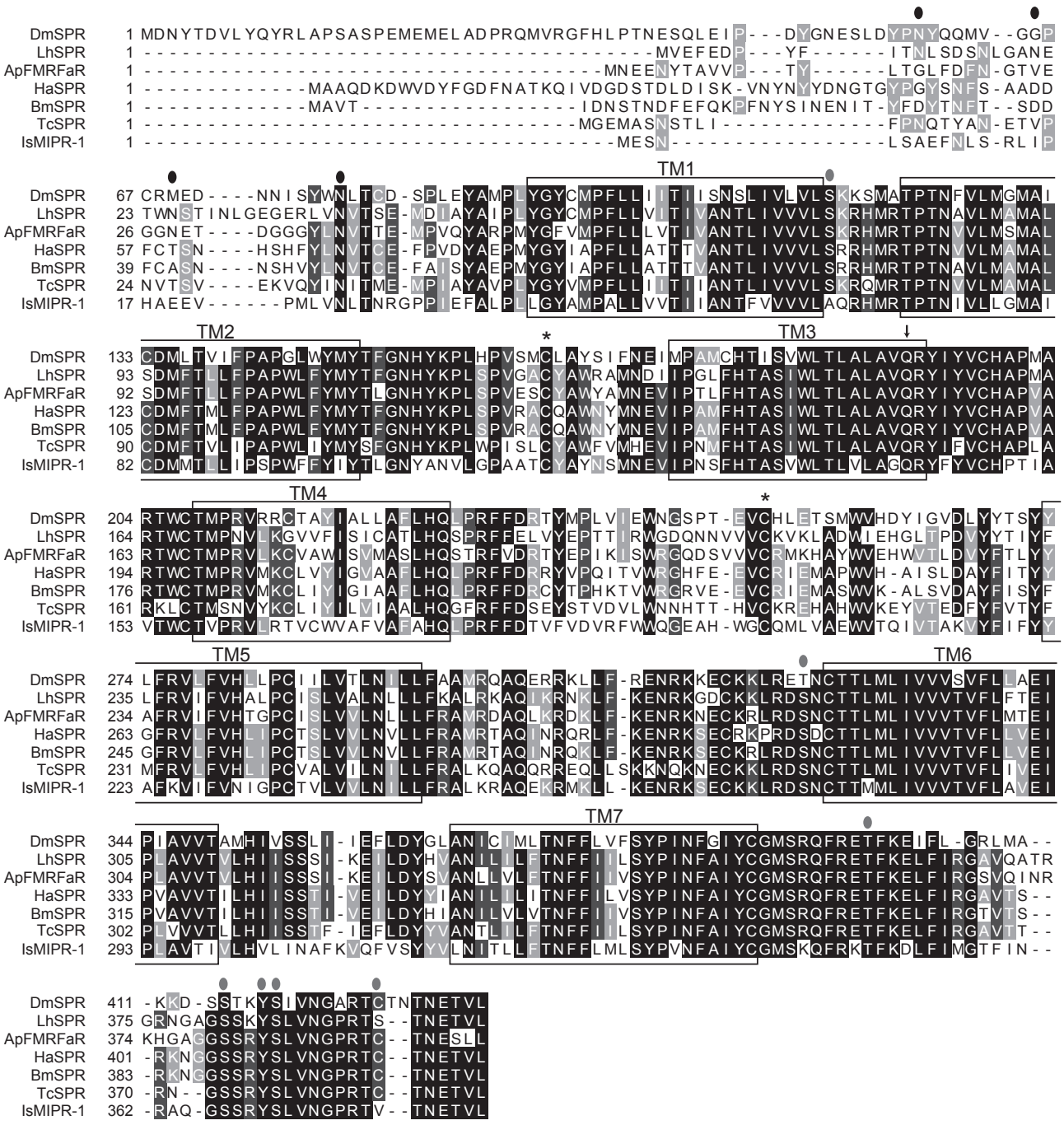

Figure 2. Amino acid sequence alignment of the *Lygus hesperus* sex peptide receptor LhSPR with sex peptide receptor (SPR)/myoinhibiting peptide receptor (MIPR) orthologues in various arthropods. Alignment was performed using the L-INS-I strategy in MAFFT (Katoh *et al.*, 2005) and rendered with JALVIEW (Waterhouse *et al.*, 2009). The grey scale amino acid identity shading is defined as: black – identity in six of the seven species; dark grey – identity in five of the seven species; and light grey – identity in at least three of the seven species. Positions of the putative transmembrane domains (boxes) are based on consensus predictions for the LhSPR sequence. Conserved Cys residues in extracellular loops 1 and 2 that probably form a stabilizing disulphide bond are marked with asterisks. The Gln mutation in the canonical G protein-coupled receptors E/DRY motif is indicated by an arrow. Putative *N*-glycosylation sites (consensus Asn-X-Ser/Thr) are indicated by black circles and putative phosphorylation sites (NetPhos2.0 server) are indicated by grey circles. Species abbreviations: DmSPR, *Drosophila melanogaster* (NP_572225); LhSPR, *Lygus hesperus* (AEK80439); ApFMRFaR, *Acyrthosiphon pisum* (XP_001944453); HaSPR, *Helicoverpa armigera* (ADK79103); BmSPR, *Bombyx mori* (NP_001108346); TcSPR, *Tribolium castaneum* (EFA01285); IsMIPR-1, *Ixodes scapularis* (XP_002400964).

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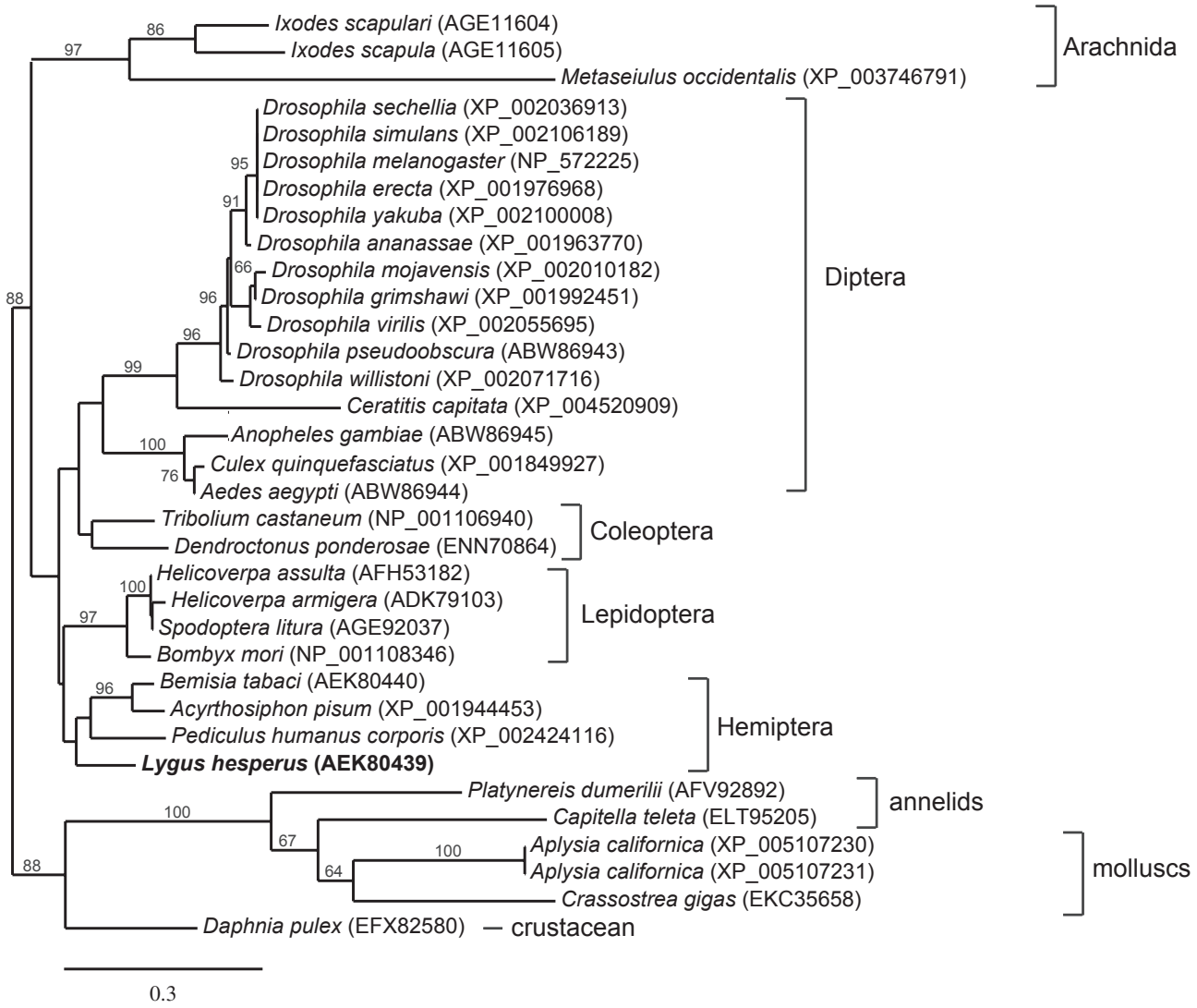

Figure 3. Neighbour-joining tree of putative sex peptide receptor (SPR)/ myoinhibiting peptide receptor (MIPR) orthologues from various metazoans. Phylogentic analysis was performed on the phylogeny.fr server (Dereeper *et al*., 2008) using *Lygus hesperus* SPR (LhSPR) with 33 additional sequences identified in a BLAST analysis. Alignment was performed using default settings for MUSCLE v3.7 followed by automatic curation with default settings in Gblocks. The phylogenetic tree was generated using the neighbour-joining method implemented in BioNJ (Gascuel, 1997) with the JTT substitution model and bootstrap support for 1000 iterations. Graphical representation of the phylogenetic tree was generated using TreeDyn with bootstrap support >60% shown. Accession numbers are indicated in parenthesis to the right of each species with the representative taxonomic Order of arthropods and general classifications of non-arthropods indicated. The LhSPR sequence is shown in bold.

performed using cDNAs prepared from the respective reproductive tissues (lateral and medial accessory glands, testis, ovary and seminal depository) as well as sex-specific heads, bodies, midgut/hindgut, and Malpighian tubules. LhSPR was amplified from all of the tissues examined, albeit to varying levels (Fig. 4B). To obtain a more accurate determination of transcript levels, we compared the expression level of LhSPR in the same sex-specific tissue sets relative to expression in either virgin female or male bodies and normalized to the internal control gene, *Lygus* actin. A significant difference in transcript levels, compared with female whole bodies, was observed in female heads (~4.8-fold higher expression; $P < 0.05$), female gut (~5.8-fold higher expression; $P < 0.01$), and seminal depository (~7-fold higher expression; $P < 0.01$) (Fig. 4C). Amongst the male tissues, only the male gut exhibited significantly higher expression (~4-fold; $P < 0.05$) compared with whole bodies (Fig. 4D). We also examined the effect of mating status on LhSPR expression. Females 24 h post-mating exhibited a significant ($P < 0.01$) decrease in LhSPR expression compared with virgin females of the same age and cohort (Fig. 4E). No difference was observed between mated and virgin males (Fig. 4E).

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Transient expression and ligand binding

To further characterize the potential function of LhSPR, we sought to examine cell surface localization and ligand specificity of the putative receptor using a heterologous expression system. Insect expression plasmids harbouring either the LhSPR coding sequence with the fluorescent reporter gene, Venus (Nagai *et al*., 2002), fused in-frame at the carboxyl terminus (LhSPR-Venus) or the reporter gene alone were constructed and used to transfect cultured *Trichoplusia ni* insect cells. Live cell imaging of construct expression was examined 48 h post-transfection using a fluorescent microscope. Cells transfected with Venus alone exhibited robust fluorescence throughout the cytosol (Fig. 5B). In contrast, fluorescence in cells expressing LhSPR-Venus was largely localized to the cell surface (Fig. 5C). No fluorescence was observed in non-transfected cells (Fig. 5A). These results confirm the predicted plasma membrane localization of LhSPR and are consistent with its putative function as a GPCR.

SPR orthologues from multiple species are activated by DmSP (Yapici *et al*., 2008; Kim *et al*., 2010; Poels *et al*., 2010). The post-mating effect of DmSP is localized to the carboxyl terminal end of the peptide (Kubli, 2008), suggesting that the amino terminal portion is dispensable for SPR binding. Indeed, a truncated analogue of DmSP corresponding to residues 21–36 (DKWCRLNLGPAWGGRC; $DmSP_{21–36}$) is comparable in efficacy to the full-length peptide (Kim *et al*., 2010). Building on these findings, we sought to examine the potential ligand specificity of LhSPR using synthetic $DmSP_{21–36}$ tagged at its amino terminus with carboxytetramethylrhodamine (TAMRA), a fluorophore that emits in the red spectrum. To validate our method, we assessed the specificity of the TAMRA-$DmSP_{21–36}$ ligand. Similar to LhSPR, we constructed a fluorescent chimera of DmSPR tagged at its carboxyl terminus with Venus (DmSPR-Venus) and examined its localization and binding capability in *T. ni* cells following 1 h incubation at 4°C. The low temperature incubation was done to minimize ligand-induced internalization (Chow *et al*., 1998), a specific cellular process that clears bound ligand from the cell surface and which is associated with receptor desensitization (Ferguson, 2001; Kristiansen, 2004). Similar to LhSPR-Venus, DmSPR-Venus localized to the cell surface in transfected cells with no evidence of fluorescence emission in the red spectrum (Fig. 6A). Incubation with 50 nM TAMRA-$DmSP_{21–36}$ resulted in robust red fluorescence that colocalized with DmSPR-Venus at the cell surface (Fig. 6C). No localized red fluorescence was seen on the surfaces of cells transfected with Venus alone (Fig. 6B), indicating that ligand binding was specific to the expressed receptor. Similar incubation with the added presence of 1 μM unlabelled $DmSP_{21–36}$ resulted in a drastic reduction in red fluorescence (Fig. 6D) and the appearance of numerous intracellular green fluorescent punctae. The presence of the punctae is consistent with internalized SPR following ligand-induced endocytosis. The addition of 1 μM synthetic *Helicoverpa zea* PBAN, an unrelated peptide characterized by a FxPRLamide carboxyl terminus (Rafaeli, 2009), had no effect on TAMRA-$DmSP_{21–36}$ binding or the appearance of internalized DmSPR-Venus (Fig. 6E). To confirm the cellular functionality of the fluorescently labelled ligand, we performed time course imaging of cells transfected with DmSPR-Venus to assess ligand-induced internalization as an indirect indicator of receptor binding and activation. As before, the initial TAMRA-$DmSP_{21–36}$ red fluorescent signal colocalized at the cell surface with DmSPR-Venus (Fig. 6F). Maintenance of the cells at 27°C for 35 min, however, resulted in a dramatic redistribution of the red fluorescent signal from the cell surface to intracellular punctae that colocalized with intracellular DmSPR-Venus (Fig. 6G). The absence of the red fluorescent punctae in non-DmSPR-Venus-expressing cells (data not shown) strongly suggests that internalization is SPR-dependent. Taken together, these results indicate that the interaction between TAMRA-$DmSP_{21–36}$ and DmSPR-Venus is specific and reversible and that the fluorescent ligand is fully functional with respect to receptor activation.

We next examined the ligand specificity of LhSPR-Venus for TAMRA-$DmSP_{21–36}$. Despite conditions identical to those used with the DmSPR-Venus assays, cells expressing LhSPR-Venus showed no evidence of TAMRA-$DmSP_{21–36}$ binding (Fig. 6H). To further validate our approach, we constructed a third fluorescent chimera using the SPR from *H. armigera* (i.e. HaSPR), which is the only other SPR shown to play a role in mediating post-mating responses (Hanin *et al*., 2012). Furthermore, virgin *H. armigera* injected with synthetic DmSP and $DmSP_{21–36}$ exhibit decreased pheromone production (Fan *et al*., 1999, 2000) and male *H. armigera* accessory glands are immunoreactive to a DmSP antibody (Nagalakshmi *et al*., 2004). These findings support the biological relevance of HaSPR and thus its utility in this assay. HaSPR-Venus localized to the cell surface (Fig. 6I) and, like DmSPR-Venus, bound TAMRA-$DmSP_{21–36}$ (Fig. 6I). The same binding profile for the three SPR constructs was observed when the concentration of TAMRA-$DmSP_{21–36}$ was increased to 1 μM (data not shown).

Addition of an epitope/reporter sequence such as Venus to a protein can adversely affect its normal function, either through steric hindrance of normal protein interactions or via occlusion of critical targeting sequence motifs (Snapp, 2009). To determine if the addition of the reporter protein to LhSPR was contributing to the impaired binding,

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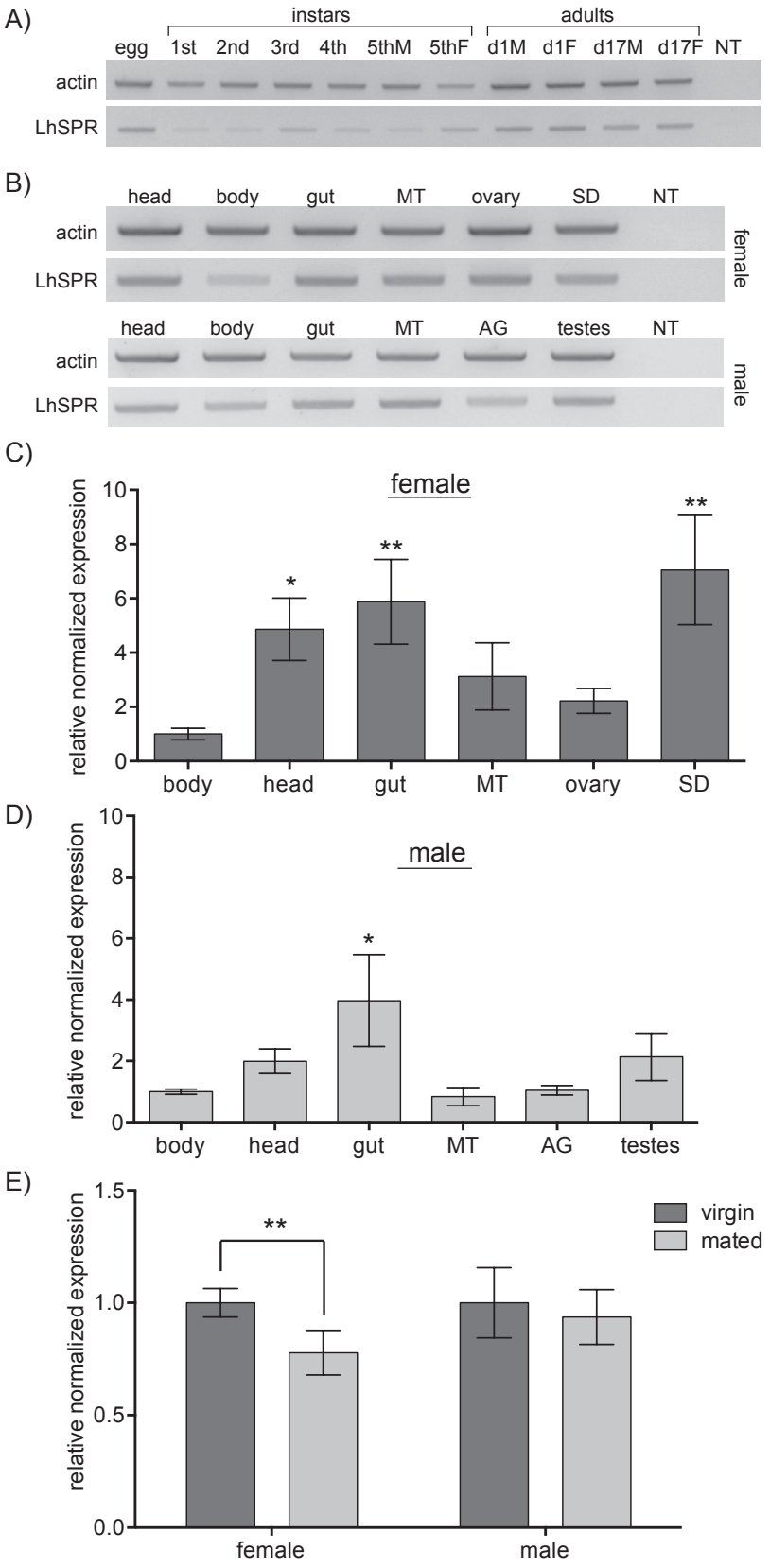

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Figure 4. *Lygus hesperus* sex peptide receptor (LhSPR) expression profile. A) Developmental expression of LhSPR from egg to 17-day-old adult stage. Abbreviations: 5thM, fifth instar male; 5thF, fifth instar female; d1M, 1-day-old adult male; d1F, 1-day-old adult female; d17M, virgin 17-day-old adult male; d17F, virgin 17-day-old adult female; NT, no template control. B) Expression of LhSPR in virgin adult male and female tissues. Abbreviations: gut, midgut/hindgut; MT, Malpighian tubules; SD, seminal depository; AG, accessory glands (lateral and medial); NT – no template control. In both (A) and (B) the LhSPR band corresponds to amplification of nt 1–495 of the LhSPR open reading frame. Amplification of a fragment (nt 1–555) of *Lygus* actin was included as a control. Products were analysed on 1.5% agarose gels and stained with SYBR Safe. For clarity, the negative images of the gels are shown. C) Relative expression level of LhSPR in virgin adult female tissues. Expression is relative to *Lygus* actin and calibrator tissue for normalization was virgin adult *L. hesperus* female whole bodies. Abbreviations: gut, midgut/hindgut; Mt, Malpighian tubules; SD, seminal depository. D) Relative expression level of LhSPR in virgin adult male tissues. Expression is relative to *Lygus* actin and calibrator tissue for normalization was virgin adult *L. hesperus* male whole bodies. Abbreviations: gut, midgut/hindgut; Mt, Malpighian tubules; AG, accessory glands (lateral and medial). E) Relative expression level of LhSPR in mated and virgin females and males. Expression is relative to *Lygus* actin and calibrator tissues for normalization were whole bodies of sex-specific virgins. In (C–E) samples were analysed in triplicate with assays repeated with three biological replicates. Error bars represent SEM values computed across replicates. Significant differences were determined using ANOVA with Tukey's multiple comparison test. A single asterisk (*) indicates $P \leq 0.05$ whereas double asterisks (**) indicate $P \leq 0.01$.

we generated and expressed constructs encoding LhSPR and DmSPR lacking the carboxyl terminal reporter. Cells transfected with DmSPR exhibited cell surface-associated red fluorescence as before, whereas no signal was detected in cells transfected with LhSPR (data not shown). The lack of TAMRA-DmSP$_{21–36}$ fluorescence could be an indication of impaired LhSPR trafficking to the plasma membrane. There is little evidence, however, in cells expressing LhSPR-Venus for either endoplasmic reticulum retention (diffuse perinuclear fluorescence) or lysosomal accumulation of misfolded protein (presence of relatively large fluorescent intracellular punctae in the absence of ligand).

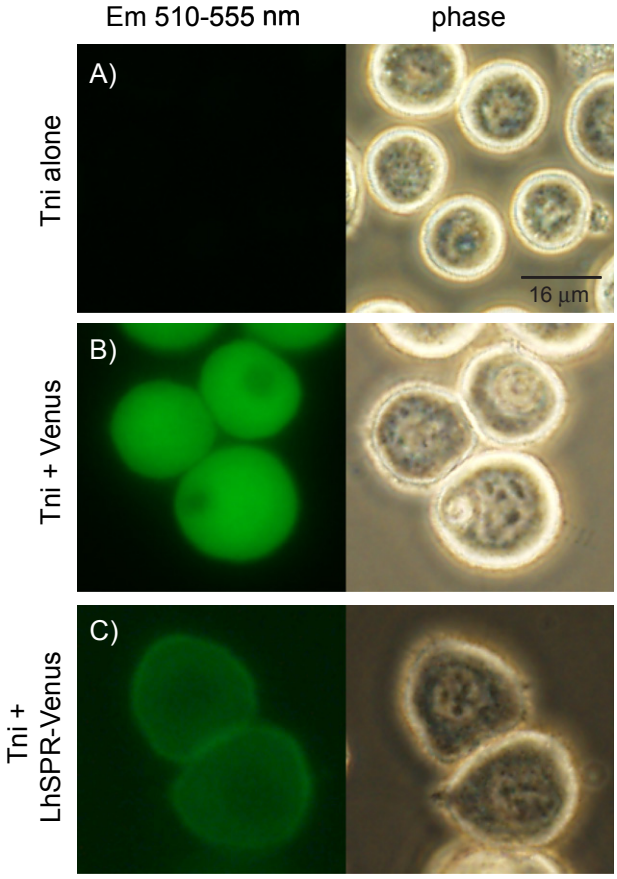

Figure 5. Cell surface localization of transiently expressed *Lygus hesperus* sex peptide receptor (LhSPR) in cultured *Trichoplusia ni* insect cells. Live cell imaging of (A) *T. ni* cells alone, (B) *T. ni* cells transfected with plasmid DNA encoding Venus alone, or (C) *T. ni* cells transfected with plasmid DNA encoding chimeric LhSPR-Venus. Images are representative of >50 cells encompassing multiple independent transfections. Scale bar (16 µm) is shown in the bottom right of panel A.

SPRs have been shown to also bind multiple MIPs (Kim *et al.*, 2010; Poels *et al.*, 2010; Yamanaka *et al.*, 2010; Šimo *et al.*, 2013). DmMIP4 (DQWQKLHGGW) is a 10-amino acid peptide shown to have a lower EC$_{50}$ (half-maximal effective concentration) value than DmSP on a number of SPRs (Poels *et al.*, 2010). To assess the binding capability of LhSPR for this class of peptides, we purchased synthetic DmMIP4 tagged at its amino terminus with TAMRA. Similar to that seen with the fluorescent DmSP analogue, both DmSPR-Venus and HaSPR-Venus bound 50 nM TAMRA-DmMIP4 (Fig. 6K, M). No binding was observed in cells expressing Venus alone (Fig. 6J) or LhSPR-Venus (Fig. 6L). As before, increasing the concentration of the ligand had no appreciable effect, and no evidence of binding was observed in cells expressing LhSPR-Venus (data not shown).

In vivo *effects of DmSP injection*

Injection of homogenized *L. hesperus* spermatophores into virgin females triggers post-mating responses similar to those of mated females (Brent, 2010a). Injection of male reproductive tissue homogenates into *H. armigera* females had a similar effect (Nagalakshmi *et al.*, 2004) as did injection of synthetic DmSP and truncated fragments of DmSP (Fan *et al.*, 1999, 2000). Although we failed to observe SP-LhSPR binding in our heterologous system (see previous section), it is possible that a factor structurally similar to SP may mediate the post-mating behavioural switch in *L. hesperus* through a different a receptor system. To examine this possibility, we injected 7- to 8-day-old adult virgin *L. hesperus* females with a 0.5-µl aliquot of either insect saline, 10 µM unlabelled synthetic DmSP$_{21–36}$, or homogenized accessory glands (combined lateral and medial glands) from virgin males aged 6–8 days. The amount of unlabelled DmSP$_{21–36}$ used in these experiments

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was comparable to that reported to have an effect in *H. armigera* females (Fan *et al*., 2000). Effects on female receptivity were assayed 24 h post-injection based on the presence or absence of a spermatophore signifying mating status. While the number of females that mated following injection with the accessory gland homogenate was comparatively low (26 out of 67; $\chi^2 = 6.169$; df = 2, adjusted $P = 0.046$), there were comparable high numbers of mated females in the $DmSP_{21–36}$ (37 out of 66) or saline (37 out of 63) injected groups, suggesting that $DmSP_{21–36}$ had no effect on *L. hesperus* mating. The lack of a behavioural effect does not appear to be attributable to injecting a non-functional peptide. Numerous cytosolic green fluorescent punctae were observed in cells transiently expressing DmSPR-Venus following the addition of unlabelled $DmSP_{21–36}$ but not HzPBAN (Fig. 6D, E). Such expression is consistent with ligand-induced receptor internalization, showing $DmSP_{21–36}$ was functional.

Discussion

Male induced post-mating changes in *D. melanogaster* and *H. armigera* are mediated by SPR (Yapici *et al*., 2008; Hanin *et al*., 2012). Because *L. hesperus* females undergo post-mating changes similar to those induced by DmSP (Strong *et al*., 1970; Brent, 2010a,b; Brent *et al*., 2011), we hypothesized that a similar ligand-receptor pair may be involved. SP, however, is poorly represented in insect genomes outside of *Drosophila*. We consequently focused our efforts on identifying the *L. hesperus* SPR orthologue. Using conserved regions of SPRs from various insect orders as a guide, we identified an SPR-like transcript (Fig. 1) with features characteristic of GPCRs and high sequence conservation (>60% identity) with functionally validated SPRs.

Spatial and temporal transcript expression profiles can provide valuable insights regarding putative gene functionality. In *Drosophila*, DmSPR is highly expressed in the female reproductive tract, in particular the spermatheca, as well as the brain and ventral nerve cord (Yapici *et al*., 2008). This expression profile correlates well with the distribution of putative SP binding sites identified previously (Ottiger *et al*., 2000; Ding *et al*., 2003). Hanin *et al*. (2011) reported similar elevated expression of HaSPR in the spermatheca and female ventral nerve cord. We likewise found that LhSPR transcripts were highly abundant in female heads and seminal depositories (Fig. 4C). Like other accessory gland proteins/peptides (Lung & Wolfner, 1999), SP is thought to pass from the female reproductive tract into the haemolymph to act directly on specific neuronal targets in the central nervous system (CNS). Indeed, expression of SPR in the CNS is essential for the SP-mediated post-mating switch; neurons that terminate in the suboesophageal ganglion are thought to be important in regulating female receptivity, whereas neurons in the abdominal ganglia are thought to be involved in egg release (Kvitsiani & Dickson, 2006; Häsemeyer *et al*., 2009; Yang *et al*., 2009; Ferveur, 2010). Unlike expression in the CNS, the functional role of SPR expression in the spermatheca, which functions in sperm storage (Qazi *et al*., 2003), has not been fully elucidated; however, interactions between stored sperm and the amino terminus of DmSP may serve as a potential repository for the peptide (Liu & Kubli, 2003). It has been suggested that as stored sperm moves from the spermatheca into the reproductive tract the bound SP dissociates, crosses into the haemolymph, and acts at the target neuronal sites (Kubli, 2003). This continuous infusion is thought to drive the long-term effects on female receptivity. Consequently, a potential role for SPR in the spermatheca may involve sequestration and/or feedback regulation, such that SP levels are high in virgin females but decrease in response to mating. Consistent with this speculation, SPR levels in the *H. armigera* spermathecae are reduced following mating (Hanin *et al*., 2011). Intriguingly, we also observed a significant decline in LhSPR levels in mated females compared with virgin females (Fig. 4E); however, what role, if any, this receptor may have in mediating Lygus reproductive behaviour remains to be determined.

While SPR has a clear role in reproductive regulation in some species (i.e. *D. melanogaster* and *H. armigera*), SPR sequence conservation across multiple genomes (exception Hymenoptera) that lack an orthologous SP suggest the presence of alternative ligands. Activation of heterologously expressed DmSPR and SPR homologues (*B. mori*, *Ae. aegypti*, *T. castaneum* and *Ixodes scapularis*)

Figure 6. Fluorescent ligand binding in *Trichoplusia ni* cells transiently expressing sex peptide receptor (SPR) chimeras. Live cell imaging of cells expressing DmSPR-Venus (A). Competitive binding assay of cells expressing either Venus (B) or DmSPR-Venus (C–E). Cells were imaged following incubation for 1 h at 4°C with 50 nM carboxytetramethylrhodamine (TAMRA)-$DmSP_{21–36}$ alone (B and C) or in the presence of 1 μM unlabelled $DmSP_{21–36}$ (D) or 1 μM unlabelled HzPBAN (E). Time-dependent internalization of TAMRA-$DmSP_{21–36}$ in *T. ni* cells transiently expressing DmSPR-Venus (F and G). Images are of the same set of cells taken immediately after the 1 h incubation (F) and following maintenance at 27°C for 35 min (G). Colocalization of the green and red fluorescence signals within the cells is indicative of ligand-mediated internalization following receptor activation. Live cell imaging of cells expressing LhSPR-Venus (H) or HaSPR-Venus (I) with 50 nM TAMRA-$DmSP_{21–36}$. Note – TAMRA-$DmSP_{21–36}$ binding is not evident in cells expressing LhSPR-Venus. Live cell imaging of cells expressing Venus (J), DmSPR-Venus (K), LhSPR-Venus (L), or HaSPR-Venus (M) following incubation as before with 50 nM TAMRA-DmMIP4. Ligand binding is apparent only in the DmSPR-Venus and HaSPR-Venus cells. Venus derived fluorescence was observed with a 510–555 nm emission filter and TAMRA-derived fluorescence was observed with a >575 nm emission filter. Colocalization of the two signals suggestive of receptor-dependent binding is depicted as yellow in the merged images. Images are representative of >50 cells encompassing multiple independent transfections.

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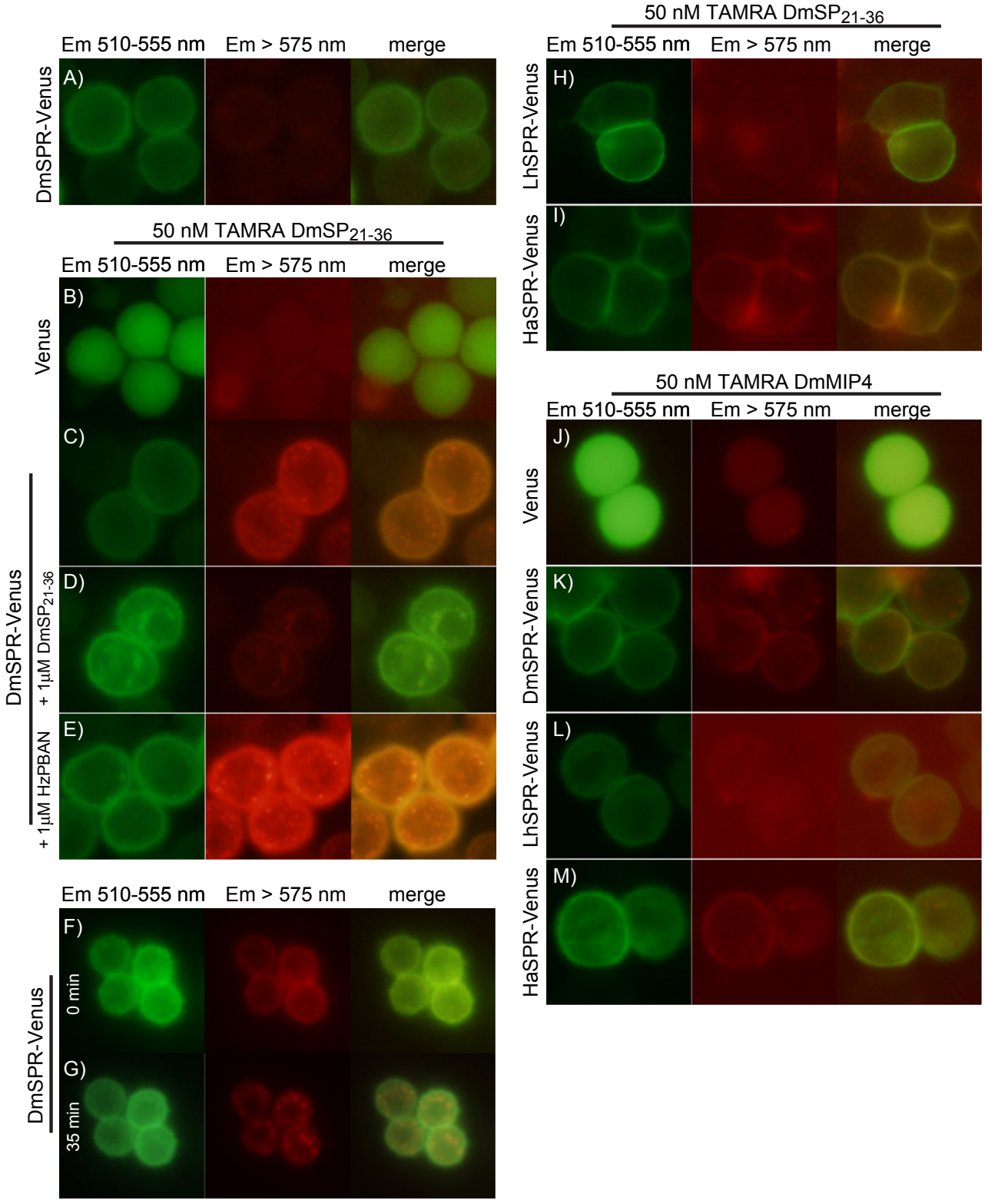

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by endogenous MIPs and observed cross-reactivity amongst the receptors led multiple groups to conclude that MIPs comprised the ancestral SPR ligand (Kim *et al.*, 2010; Poels *et al.*, 2010; Yamanaka *et al.*, 2010; Šimo *et al.*, 2013). MIPs, however, have no effect on the post-mating female response (Kim *et al.*, 2010), implying SPR involvement in other biological functions. Consistent with a novel non-reproductive role, SPR and SPR-like transcripts and/or expression have been reported in multiple arthropod tissues and stages: *D. melanogaster* male CNS (Yapici *et al.*, 2008), male *H. armigera* reproductive tissues and CNS (Hanin *et al.*, 2011), *B. mori* fifth instar larval prothoracic glands and Malpighian tubules (Yamanaka *et al.*, 2010), *I. scapularis* salivary glands (Šimo *et al.*, 2013), and across *D. melanogaster* developmental stages including eggs (Poels *et al.*, 2010). We also observed LhSPR expression across the *L. hesperus* nymphal developmental stages (i.e. first to fifth instars) and eggs (Fig. 4A) as well as male reproductive tissues and tissue comprising the midgut/hindgut and Malpighian tubules from both sexes (Fig. 4B). This varied profile suggests SPR is probably a pleiotropic receptor that mediates multiple biological functions. Indeed, MIP-mediated activation of SPR has been linked to regulation of ecdysteroid biosynthesis in *B. mori* (Yamanaka *et al.*, 2010) and salivary gland activity in *I. scapularis* (Šimo *et al.*, 2013). In various species, MIPs have been implicated in diverse functions including inhibition of various gland and muscle processes including juvenile hormone production (Lorenz *et al.*, 1995), ecdysteroid synthesis (Hua *et al.*, 1999; Yamanaka *et al.*, 2010), and spontaneous visceral muscle contractions (Schoofs *et al.*, 1991; Blackburn *et al.*, 1995, 2001; Predel *et al.*, 2001). Within the CNS, MIPs have been proposed to have roles in sensory processing (Carlsson *et al.*, 2010; Schulze *et al.*, 2012), the circadian clock (Kolodziejczyk & Nässel, 2011a, 2011b; Schulze *et al.*, 2012), and ecdysis behaviours (Davis *et al.*, 2003; Kim *et al.*, 2006a, 2006b). In addition, MIP immunoreactivity within the hindgut/midgut of numerous species (Schoofs *et al.*, 1996; Predel *et al.*, 2001; Williamson *et al.*, 2001; Davis *et al.*, 2003; Veenstra, 2009; Reiher *et al.*, 2011; Lange *et al.*, 2012) suggests a potential role in gut motility. Consistent with this proposed role, LhSPR expression was highest in the guts of both males and females (Fig. 4C, D). The processes regulated by MIPs in *L. hesperus*, a piercing-sucking insect that must process a large quantity of watery food, and the potential involvement of LhSPR in mediating intestinal physiology remain to be elucidated, but are expected to form the basis of future studies.

While the primary structures of DmSP and MIPs share little similarity, both are characterized by a carboxyl terminal tryptophan (Trp; W) motif. The larger SP (36 amino acid) has a W-X_8-Wamide motif (i.e. Trp23 and Trp32), whereas most of the 9–12 amino acid MIPs exhibit a W-X_6-Wamide motif. Full functionality of the respective ligands requires the presence of both residues (Kim *et al.*, 2010; Poels *et al.*, 2010). Molecular modelling predictions of $DmSP_{21–36}$ and DmMIP1 suggested that both conformations adopt a β-turn stabilized by the Trp residues (Kim *et al.*, 2010). NMR studies of the full-length DmSP solution structure confirmed the β-turn conformation and further revealed that Trp23, Leu26, Leu28 and Trp32 form a hydrophobic patch that may be involved in receptor binding (Moehle *et al.*, 2011). Consequently, given the significant sequence similarity of the respective receptors, the inability of LhSPR to bind fluorescent analogues of either DmSP or DmMIP4 was surprising. One possible explanation is that the extra bulk from addition of the fluorescent tag to the amino termini blocked interactions with the ligand pocket. Clear colocalization of the analogues with the control receptors (i.e. DmSPR and HaSPR) and internalization of bound ligand in cells expressing DmSPR, however, suggests that impaired binding was not the result of steric hindrance. Consequently, the defect appears to be intrinsic to the LhSPR sequence. A possible methodological explanation is that heterologous expression of LhSPR in the *T. ni* cultured cell line results in the generation or loss of post-translational modifications that destabilize the LhSPR binding pocket without affecting cell surface trafficking. Future studies using other expression systems are expected to resolve this issue. An alternative explanation for the impaired binding is that the conformation of the respective SPR binding pockets may have changed over time with the hemipteran LhSPR diverging from that of the two holometabolous SPRs. Given receptor-ligand co-evolution, deviations in the binding pocket would be expected to be mirrored by structural changes in the ligand as well. MIPs have been identified from a host of arthropods (Blackburn *et al.*, 1995, 2001; Lorenz *et al.*, 1995; Hua *et al.*, 1999; Predel *et al.*, 2001; Williamson *et al.*, 2001; Li *et al.*, 2008; Weaver & Audsley, 2008; Šimo *et al.*, 2009) with almost all characterized by the canonical W-X_6-Wamide motif. Intriguingly, amongst a number of hemipterans, however, MIPs have been found that exhibit a W-X_7-Wamide motif (Christie, 2008; Huybrechts *et al.*, 2010; Ons *et al.*, 2011; Lange *et al.*, 2012). In the aphids *Acyrthosiphon pisum*, *Aphis gossypii*, and *Myzus persicae*, the MIP precursors encoded seven, five and seven MIPs respectively with two of the peptides from each species exhibiting the W-X_7-Wamide motif. Similarly, of the 12 MIP isoforms in *Rhodnius prolixus*, nine have the unique spacing pattern. Functional analysis revealed that the W-X_7-Wamide isoform was more potent in *R. prolixus* hindgut inhibition assays but was 100-fold less effective at activating recombinant DmSPR (Lange *et al.*, 2012). A search of our adult *L. hesperus* transcriptome (Hull *et al.*, 2013) revealed a MIP precursor sequence, translation of which would yield a 357-amino acid polypeptide with an

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MLQLIITILVGLATLACTQCYDNNKVRSLPDTNEEDRDAKVVKRSTECGNTGWQDPKSDSEKRA  64
WNNMQATWGKRKWQDMQNPGWGKRTPTPPEFDKDIQASLQTSFQEDKRGWKDMQGPSWGK  124
RGWQDMRQPSWGKRGWKDMQTSAWGKRGWQDMQGQWGKRGWQDMQGNWGKRGWQDMQ  181
GLNWGKRRWQDMPTSAWGKRGWKELQTTGWGKRDALEDQMADKRGWGDMKISGYGKRAWN  241
DIQSSGWGKRGWKEMPSTGWGKRSPQDHEDFDDLEEDWELPSKSEVDIEDLLSAAEWPLGKE  303
EGANRNDRHLYEPLDDQALFGKILEGLYKASWTNRNFNPKNLAEEAGTSAQRNR  357
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Figure 7. Predicted amino acid sequence of the putative *Lygus hesperus* myoinhibiting peptide (LhMIP) precursor. The predicted signal peptide (SignalP) is shown boxed. Canonical dibasic cleavage sites (Lys-Arg) are overlined and predicted α-amdiation sites (Gly) are asterisked. Putative mature LhMIPs with a W-X_6-Wamide motif are highlighted in dark grey, those with a W-X_7-Wamide motif in medium grey, and the MIP-like sequence containing a Tyr substitution of the second Trp is shown in light grey. GenBank accession number KF697189.

amino terminal signal peptide and 17 Lys-Arg pro-hormone convertase target sites (Fig. 7). Cleavage, carboxypeptidase removal of dibasic pairs, and subsequent α-amdiation of exposed Gly residues would yield three W-X_6-Wamide MIPs, nine W-X_7-Wamide MIPs and a MIP-like peptide (Tyr substitution of the 2nd Trp) with a W-X_7-Yamide spacing. Similar to that proposed by Lange *et al*. (2012), we speculate that co-evolution of the receptor-ligand pairs may have resulted in LhSPR having higher affinity for the W-X_7-Wamide motif. This potentially could explain the lack of binding we observed following incubation with TAMRA-DmSP$_{21–36}$ (W-X_8-Wamide) and TAMRA-DmMIP4 (W-X_6-Wamide). In contrast, the promiscuity of DmSPR, a MIPR that has been co-opted by DmSP, suggests that a more accommodating binding pocket was evolutionarily selected for in Drosophila. This divergence may explain the lack of a biological effect on *L. hesperus* female receptivity following injection of unlabelled DmSP$_{21–36}$. Other possibilities include the the injected peptide being quickly rendered inactive following injection, the inability of the peptide to reach the target site, or the behavioural switch being mediated by a different ligand-receptor pair.

In conclusion, we have identified a GPCR from *L. hesperus* that has high sequence conservation with known SPR/MIPRs, and is expressed in a number of tissues that would be consistent with a role in mediating female reproductive behaviour and/or inhibition of hindgut/midgut muscle contractions. The receptor, which we designated LhSPR based on sequence similarity, localizes to the cell surface of plasmid transfected cultured insect cells but unlike other SPRs does not bind fluorescent analogues of DmSP or DmMIP. We speculate that the LhSPR binding pocket may have diverged over evolutionary time to better accommodate the W-X_7-Wamide motif that predominates amongst the predicted LhMIPs. Given the limited resources previously available for dissecting the molecular mechanisms driving *Lygus* physiology, the present study is expected to provide the basis for future research into the functions mediated by LhSPR and the role MIPs may have in regulating *Lygus* gut motility.

Experimental procedures

Insect rearing

Lygus hesperus used in the present study were from a laboratory colony maintained in-house (USDA-ARS Arid Land Agricultural Research Center, Maricopa, AZ, USA) that undergoes periodic outbreeding with locally caught conspecifics. Insect-rearing conditions consisted of 25 °C under 20% humidity with a 14 h light: 10 h dark photoperiod and a diet comprising green beans and an artificial diet mix (Debolt, 1982) in disposable packs as described by Patana (1982).

Lygus hesperus *SPR gene cloning*

To facilitate identification of the *L. hesperus* SPR sequence, degenerate primers (Table 2) were designed to conserved amino acids (TFGHNHYKP and YIYVCHAP) present in SPR sequences from *D. melanogaster*, *Ae. aegypti*, *B. mori*, and *T. castaneum*. Based on high DmSPR expression in female reproductive tissue (Yapici *et al*., 2008), reproductive tissue (ovary, oviduct, ovariole and seminal depository) from 20 7-day-old adult *L. hesperus* virgin females was dissected and total RNA isolated using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA). First-strand cDNA was generated from 2 μg total RNA using random hexamers and Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Degenerate PCR was performed using 0.7 μl cDNA and 5 μl of each 10 μM degenerate primer (Table 2) with ExTaq DNA polymerase (Takara Bio Inc./Clontech, Madison, WI, USA). Thermocycler conditions consisted of 95 °C for 2 min followed by 10 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, then 15 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, 20 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s, and finished with a 5-min incubation at 72 °C. An aliquot (1 μl) of the PCR products was then used as a template for a second round of amplification using the same primer set and thermocycler conditions consisting of 95 °C for 2 min, followed by 10 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, then 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, and finished with a 5-min incubation at 72 °C. PCR products were separated on a 1.5% agarose gel and visualized with SYBR Safe (Invitrogen). Amplimers of the expected size were gel-excised using an EZNA Gel Extraction kit (Omega

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Table 2. Oligonucleotide primers used in cloning and analysis of the *L. hesperus* SPR

Primer	Sequence (5′ to 3′ orientation)	Purpose
SPR deg F	CNTTYGGTAAYCAYTAYAARCC	degenerate PCR
SPR deg R	NGGNGCRTGRCANACRTADATRTA	degenerate PCR
LhSPR RACE F1	CCTGGTCTATTCCATACAGCCTCG	RACE PCR
LhSPR RACE R1	CGAGGCTGTATGGAATAGACCAGG	RACE PCR
LhSPR start F	AATATGGTTGAGTTTGAGGAC	amplify LhSPR/endpoint PCR
LhSPR end R	CAGAACAGTCTCGTTTGTAC	amplify LhSPR
DmSPR start F	GAGATGGACAACTATACGGA	amplify DmSPR
DmSPR end R	GAGGACCGTCTCGTTGG	amplify DmSPR
HaSPR start F	ATGGCGGCGCAAGATAAAGACTGG	amplify HaSPR
HaSPR end R	AAGCACAGTTTCGTTGGTGC	amplify HaSPR
LhSPR nt 454 R	CCGCCAGTGCCAATGTCAGCC	end point PCR
Lygus actin nt 1 F	ATGTGCGACGAAGAAGTTG	end point PCR
Lygus actin nt 555 R	GTCACGGCCAGCCAAATC	end point PCR
rt LhSPR nt 1115 F	TGTGGGATGTCCCGTCAGTTCCG	real-time qPCR
rt LhSPR nt 1243 R	ACTGGTCCTCGGGCCATTCACC	real-time qPCR
rt Lh actin nt 683 F	TGGCCACCGCCGCGTCCT	real-time qPCR
rt Lh actin nt 787 R	AGAGGGCTTCGGGGCACCTG	real-time qPCR
LhSPR-Venus F	GTACAAACGAGACTGTTCTGATGGTGAGCAAGGGCGAG	overlap extension PCR
LhSPR-Venus R	CTCGCCCTTGCTCACCATCAGAACAGTCTCGTTTGTAC	overlap extension PCR
DmSPR-Venus F	CCAACGAGACGGTCCTCATGGTGAGCAAGGGCGAG	overlap extension PCR
DmSPR-Venus R	CTCGCCCTTGCTCACCATGAGGACCGTCTCGTTGG	overlap extension PCR
HaSPR-Venus F	GCACCAACGAAACTGTGCTTATGGTGAGCAAGGGCGAG	overlap extension PCR
HaSPR-Venus R	CTCGCCCTTGCTCACCATAAGCACAGTTTCGTTGGTGC	overlap extension PCR
mVenus R	CTAGGCGGCGGTCACGCGTTC	overlap extension PCR

SPR, sex peptide receptor; Lh, *Lygus hesperus*; Ha, *Helicoverpa armigera*; RACE, rapid amplification of cDNA ends; Dm, *Drosophila melanogaster*.

Bio-Tek Inc., Norcross, GA, USA), cloned into pGEM-TE cloning vector (Promega, Madison, WI, USA), and sequenced.

To obtain the 5′ and 3′ ends of the *L. hesperus* SPR sequence, gene-specific RACE primers (Table 2) were designed using the degenerate PCR product above. RACE cDNA was generated using a SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions with ovary-derived total RNA as the first-strand template. First round RACE PCR amplification was performed using ExTaq DNA polymerase. Thermocycler conditions consisted of 95 °C for 2 min followed by five cycles at 94 °C for 30 s, 70 °C for 20 s, and 72 °C for 90 s, then five cycles at 94 °C for 30 s, 67 °C for 20 s, and 72 °C for 90 s, 30 cycles at 94 °C for 30 s, 62 °C for 20 s, and 72 °C for 90 s, and finished with a 5-min incubation at 72 °C. An aliquot (0.5 μl) of the PCR product was used as a template for second round amplification with nested primers (Table 2) and identical thermocycler conditions. PCR products were separated on a 1.5% agarose gel and visualized with SYBR Safe. Amplimers were gel-excised, subcloned, and sequenced as before.

The resulting 5′ and 3′ RACE sequence data were assembled with the internal degenerate PCR-derived fragment to generate a full-length LhSPR transcript containing an open reading frame (ORF) of the expected size. Gene-specific primers (Table 2) designed to encompass the putative start and stop codons were used in multiple independent reactions using ovary-derived cDNA as a template to confirm the LhSPR coding sequence. The consensus nucleotide sequence has been deposited with GenBank (accession number JF273642).

Bioinformatic sequence analyses

Comparison of the LhSPR gene sequence with database sequences was performed using BLASTx (http://blast.ncbi.nlm.nih.gov/). Alignment with additional arthropod SPR sequences was done using the L-INS-I strategy in MAFFT (Katoh *et al.*, 2005). The phylogeny.fr server (Dereeper *et al.*, 2008) was used to perform all phylogenetic analyses and incorporated sequences identified in BLASTX analyses with an *e* value $< e^{-65}$. Sequence alignment was performed using default settings for MUSCLE (Edgar, 2004) followed by automatic curation with Gblocks (Castresana, 2000). A phylogenetic tree was generated using the neighbor joining method implemented in BioNJ (Gascuel, 1997) with the JTT substitution model and bootstrap support for 1000 iterations. Graphical representation of the phylogenetic tree was performed with TreeDyn (Chevenet *et al.*, 2006). Subcellular localization prediction was performed using PSORTII (Horton & Nakai, 1997). Prediction of phosphorylation sites was performed using the NetPhos2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) (Blom *et al.*, 1999). Topology and transmembrane domain predictions were performed using TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) (Hofmann & Stoffel, 1993), TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh *et al.*, 2001), Phobius (http://phobius.sbc.su.SE/) (Käll *et al.*, 2007), RHYTHM (http://proteinformatics.charite.de/rhythm/) (Rose *et al.*, 2009), TOPCONS (http://topcons.cbr.su.SE/) (Bernsel *et al.*, 2009), HMMTOP v2.0 (http://www.enzim.hu/hmmtop/) (Tusnády & Simon, 2001), and TopPred II (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::toppred) (von Heijne, 1992).

PCR-based profiling of LhSPR expression

To examine the expression profile of LhSPR, total RNA was isolated from eggs, first, second, third, fourth and early fifth instars, late fifth instar males and females, as well as 1-day-old and 17-day-old virgin adult males and females. Total RNAs were also isolated from various tissues in virgin 15-day-old male and

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female adults: body (×3), head (×15), mixed hindgut/midgut (×25), Malpighian tubules (×20), accessory gland (five pairs of lateral and medial glands), testis (five pairs), ovary (five pairs), and seminal depository (×20). In addition, total RNA was also isolated from mated and virgin males/females 24 h post-mating (three of each sex in triplicate). TRI Reagent Solution (Ambion-Life Technologies, Carlsbad, CA, USA) was used for all total RNA isolations. cDNAs were generated with a Superscript III first-strand cDNA synthesis kit (Invitrogen) using random pentadecamers (IDT, San Diego, CA, USA) and 500 ng of DNase I-treated total RNAs. Initial expression profiles were carried out using end-point PCR amplification with Sapphire Amp Fast PCR Master Mix (Clontech Laboratories Inc.) and primers (Table 2) designed to amplify the first 555 nucleotides of *Lygus* actin (DQ386914) or a 454-bp fragment of LhSPR (nt 1-454). Thermocycler conditions consisted of 95 °C for 2 min followed by 35 cycles at 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s, and finished with a 5-min incubation at 72 °C. PCR products were separated on 1.5% agarose gels using a Tris/acetate/EDTA buffer system and visualized with SYBR Safe.

To further examine the differential expression profile, real-time PCR oligonucleotide primers (Table 2) designed to amplify a ~100–150-bp region of LhSPR (nt 1115–1243) or the *Lygus* actin gene (nt 683–787) were designed using Primer3 (Rozen & Skaletsky, 2000). Amplification of single discrete products using these primers with the cDNAs described above was confirmed by endpoint PCR with Sapphire Amp Fast PCR Master Mix. Real-time PCR was performed on a Bio-Rad CFX96 real-time cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a 10-μl volume using a Kapa SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA, USA) and a 1 : 2.5 dilution of the respective cDNA templates, which were derived from the sex-specific adult tissues or mated/virgin males and females described above. Three biological replicates of the cDNA templates were analysed with three technical replicates run for each cDNA set. Thermocycler conditions consisted of 95 °C for 60 s followed by 40 cycles of 94 °C for 10 s and 60 °C for 20 s. Melt curve analysis was performed at the end of each amplification run to confirm the presence of a single peak. Relative expression was determined using the Pfaffl method (Pfaffl, 2001), which incorporates the respective primer efficiencies for the target and reference genes into the standard ΔΔCt method. Primer efficiencies for LhSPR and *Lygus* actin were determined from serial dilutions of cDNA as well as linearized plasmid DNA containing the respective coding sequences. The efficiency for the LhSPR primer set was determined to be 94.9%, while that of the *Lygus* actin primer pair was 97.3%. The expression of LhSPR relative to *Lygus* actin following normalization (whole virgin female bodies for female tissues, whole virgin male bodies for male tissues, virgin females for mated females, and virgin males for mated males) was determined using Bio-Rad CFX Manager Software v3 (Bio-Rad Laboratories Inc.). Results are presented as means ± SEM with statistical analysis (ANOVA with Tukey's multiple comparison test, $P < 0.05$) performed using GraphPad Prism v6 (GRAPHPAD Software, La Jolla, CA, USA).

Construction of insect expression plasmids

To examine the cellular localization of LhSPR and ligand binding specificity, various insect expression vectors were constructed using the pIB/V5-His TOPO TA insect expression vector (Invitrogen). Chimeras of DmSPR, HaSPR, and LhSPR tagged at the carboxyl terminus with the fluorescent reporter protein Venus (Nagai *et al.*, 2002) were generated via overlap extension-PCR (Wurch *et al.*, 1998) using ExTaq DNA polymerase or KOD Hot Start DNA polymerase (Toyobo/Novagen, EMD Biosciences, San Diego, CA, USA) with gene-specific primers and plasmid DNAs encoding the various SPRs. Full-length DmSPR was amplified from Drosophila Genomic Resource Center clone #RE15579 using primers designed to encompass the ORF start and stop codons (Table 2). Full-length HaSPR was amplified using gene-specific primers (Table 2) designed to the ORF (GenBank accession HM567403) and *H. armigera* brain cDNA kindly provided by Dr A. Rafaeli (Volcani Institute, Israel). The initial overlap extension reactions were performed using a SPR-specific forward primer with a chimeric reverse and a chimeric forward primer with a Venus reverse primer (Table 2). Thermocycler conditions consisted of 95 °C for 2 min followed by 21 cycles at 95 °C for 20 s, 58 °C for 10 s, and 70 °C for 15 s with a final 5-min incubation at 72 °C. The second overlap extension reactions used aliquots of the initial reactions with an SPR-specific forward primer and the Venus reverse primer. Thermocycler conditions consisted of 95 °C for 2 min followed by 21 cycles at 95 °C for 20 s, 58 °C for 10 s, and 70 °C for 45 s with a final 5-min incubation at 72 °C. The resulting products were gel excised, treated with ExTaq DNA polymerase as necessary to add 3′A overhangs, cloned into the pIB/V5-His TOPO TA vector, and sequence verified. Non-fluorescent expression vectors encoding DmSPR and LhSPR were likewise generated using gene-specific primers and ExTaq DNA polymerase. All constructs were sequence validated before use.

Transient expression in cultured insect cells

Trichoplusia ni cells (Orbigen Inc., San Diego, CA, USA) were maintained as an adherent monolayer in serum-free insect culture media (Orbigen Inc.). *T. ni* cells seeded into 35 mm #1.5 glass bottom dishes (In Vitro Scientific, Sunnyvale, CA, USA) were transfected with 2 μg plasmid using 4 μl Insect Gene Juice transfection reagent (Novagen, EMD Biosciences, San Diego, CA, USA) or Cellfectin II (Invitrogen) for 5 h. At the end of the transfection period, the transfection media was removed and the cells were washed twice with 1 ml serum-free media. Transfected cells were maintained in serum-free media for 48 h at 28 °C. Transfected cells were washed twice with 1 ml IPL-41 insect media (Invitrogen) and imaged in 2 ml IPL-41 with an Olympus FSX-100 fluorescence microscope using FSX-BSW imaging software (Olympus, Center Valley, PA, USA). Images were processed using Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

Fluorescent microscopy examination of fluorescent-labelled ligand binding

To assess the specificity of ligand binding, *T. ni* cells transiently expressing the SPR constructs above were incubated for 1 h at 4 °C in the dark with 50 nM or 1 μM synthetic $DmSP_{21–36}$ (DKWCRLNLGPAWGGRCamide) or DmMIP4 (DQWQKLHGG-Wamide) labelled at the amino terminus with the red fluorophore, carboxytetramethylrhodamine (TAMRA; λ_{Em} = ~580 nm). Competitive binding assays using *T. ni* cells transiently expressing DmSPR-Venus were performed using 50 nM of the fluorescently tagged $DmSP_{21–36}$ with 1 μM unlabelled synthetic $DmSP_{21–36}$ or 1 μM *Helicoverpa zea* pheromone biosynthesis activating

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neuropeptide (PBAN; LSDDMPATPADQEMYRQDPEQIDSR-TKYFSPRLamide; Bachem Bioscience Inc., King of Prussia, PA, USA) for 1 h at 4 °C. In all binding experiments, a low temperature was used to minimize loss of bound ligand from the cell surface by way of receptor internalization (Chow *et al.*, 1998). At the end of the incubation, live cells were rinsed with cold IPL41 and imaged as before in 2 ml IPL-41 with an Olympus FSX-100 fluorescence microscope. Synthetic peptides were commercially custom-made at >95% purity (United Peptide, Cabin John, MD, USA).

Effect of injected DmSP on female L. hesperus *mating behaviour*

An aliquot (0.5 μl) of either insect saline (16.5 mM glucose, 20 mM KCl, 5.0 mM NaCl, 6.5 mM $NaHCO_3$, 9.5 mM Na_2HPO_4, and 1.75 mM NaH_2PO_4-H_2O; pH adjusted to 7.4 with 1 M NaOH), 10 μM unlabelled synthetic $DmSP_{21–36}$ (United Peptide), or five pairs of homogenized accessory glands dissected from virgin males aged 6–8 days was injected into the abdominal lumen using a graduated borosilicate glass syringe, as previously described (Brent, 2010a). Injected females recovered for 1 h and were then placed in a covered glass Petri dish (60 × 15 mm) with two virgin males, aged 6–8 days. For a source of nourishment, the arena also included a green bean pod segment (50 mm). Insects were allowed to interact freely for ~24 h, after which females were dissected to determine if they had been inseminated.

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