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Genomic and functional characterization of a methoprene-tolerant gene in the kissing-bug *Rhodnius prolixus*

María José Villalobos-Sambucaro [a,b], Fernando Luis Riccillo [a,b], Gustavo Mario Calderón-Fernández [a,c], Marcos Sterkel [b], Luis Anibal Diambra [b], Jorge Rafael Ronderos [a,b,*]

[a] Cátedra de Histología y Embriología Animal, (Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata), La Plata, Argentina
[b] Centro Regional de Estudios Genómicos (CREG), (Facultad de Ciencias Exactas, Universidad Nacional de La Plata), La Plata, Argentina
[c] Instituto de Investigaciones Bioquímicas La Plata (INIBIOLP), (Universidad Nacional de La Plata-CONICET), La Plata, Argentina

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ABSTRACT

Metamorphosis, which depends upon a fine balance between two groups of lipid-soluble hormones such as juvenile hormones (JHs) and ecdysteroids, is an important feature in insect evolution. While it is clear that the onset of metamorphosis depends on the decrease of JH levels, the way in which these hormones exert their activities is not fully understood in Triatominae species. The discovery of a *Drosophila melanogaster* mutant resistant to the treatment with the JH analog methoprene, led finally to the description of the methoprene-tolerant gene in *Tribolium castaneum* (*TcMet*) as a putative JH receptor. Here we present the genomic and functional characterization of an ortholog of the methoprene-tolerant gene in the hemimetabolous insect *Rhodnius prolixus* (*RpMet*). The analysis of the *R. prolixus* gene showed that the exonic structure is different from that described for holometabolous species, although all the critical protein motifs are well conserved. Expression analysis showed the presence of *RpMet* mRNA in all the tested tissues: ovary, testis, rectum, Malpighian tubules and salivary glands. When juvenile individuals were treated with *RpMet* specific double strand RNA (dsRNA), we observed abnormal molting events that resulted in individuals with morphological alterations (adultoids). Similarly, treatment of newly emerged fed females with dsRNA resulted in an abnormal development of the ovaries, with eggs revealing anomalies in size and accumulation of yolk, as well as a decrease in the amount of heme-binding protein. Altogether, our results validate that *RpMet* is involved in the transduction of JH signaling, controlling metamorphosis and reproduction in *R. prolixus*.

1. Introduction

Metamorphosis plays an important role in insect evolution facilitating the ability of these organisms to colonize different environments and generating the great diversity of known insect forms. The control of metamorphosis depends upon a fine balance between at least two groups of lipid-soluble hormones: the juvenile hormones (JHs) and ecdysteroids, and associated signaling pathways (Belles, 2011; Belles and Santos, 2014; Jindra et al., 2013). Although new evidence suggests that JHs might not be essential during the first larvae stages (Smykal et al., 2014), since the pioneering work of Sir Vincent Brian Wigglesworth (1934, 1936, 1940, 1948) it was clear that the onset of metamorphosis depends on the decrease of JHs synthesis. Furthermore, JHs are also relevant in the adult female, regulating both the synthesis of vitellogenin in extra-ovarian tissues as well as their uptake by the developing oocytes in *Rhodnius prolixus* (Coles, 1965; Wang and Davey, 1993).

Methoprene is a synthetic JH analog that efficiently mimics the activity of these hormones in several insect species, altering growth in larvae, as well as reproduction in adults (Henrick, 2007). Wilson and Fabian (1986) reported the existence of a *Drosophila melanogaster* mutant insensitive to methoprene. This methoprene-tolerant mutant was around 100 times more resistant to methoprene than the wild type, and it also had resistance to JH III (Wilson and Fabian, 1986). The *Met* gene encodes a bHLH-PAS protein that binds JH with high affinity at physiological concentrations (Charles et al., 2011). Indeed, Met is localized at the nucleus (Pursley et al., 2000), suggesting that it acts as a transcription factor (Ashok et al., 1998; Miura et al., 2005). Although the binding affinity of the Met protein for JHs was demonstrated in *D.*

* Corresponding author at: FCNyM-UNLP. Calle 64 N°3 (1900), La Plata, Argentina. Fax: +54 011 42758100.
E-mail addresses: jrondero@fcnym.unlp.edu.ar, ronderos@isis.unlp.edu.ar (J.R. Ronderos).

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melanogaster, its role as a JHs receptor could not be definitively proved (Wilson, 2004). However, the use of RNA interference methods (RNAi) to block the expression of the *Met* ortholog in *Tribolium castaneum* (*TcMet*) resulted in metamorphic alterations consistent with the lack of JH activity, suggesting that the protein encoded by *TcMet* was acting as a JH receptor (Konopova and Jindra, 2007). Furthermore, it has been recently shown that Met also mediates JH action in *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae) and *Blattella germanica* (Lozano and Belles, 2014), implying that this protein could be also acting as a JH receptor in hemimetabolous species (Konopova et al., 2011). These authors also demonstrated that Met regulates the JH-responsive anti-metamorphic gene *Krüppel-homolog 1* (*Kr-h1*), both in holometabolous and in hemimetabolous insects, including *R. prolixus* (Konopova et al., 2011; Lozano and Belles, 2011).

Wigglesworth in 1934 described the existence of an anti-metamorphic hormone in the blood of *R. prolixus* (Wigglesworth, 1934). He also indicated that the source of this "inhibitory hormone" was the *corpora allata* (CA). Although the actual form of JH synthesized by *R. prolixus* still remains to be determined, it is clear from the experiments performed by Wigglesworth, and by the effects of suppression of CA activity induced by Precocene II (Bowers et al., 1976), that JHs play a fundamental role in the control of metamorphosis in Triatominae insects (Ronderos, 2009; Stoka et al., 1988; Tarrant and Cupp, 1978).

In this study we have functionally characterized the *R. prolixus Met* gene. Using RNAi experiments the expression of *Met* was blocked, altering metamorphosis in nymphs and reproductive processes in mature females. Our studies confirmed that *R. prolixus Met* (*RpMet*) is involved in the transduction of JH signaling mediating the regulation of metamorphosis and reproduction in *R. prolixus*.

2. Materials and methods

2.1. Insects

Fourth-instar larvae and newly emerged adult females of *R. prolixus* were obtained from a colony maintained at 28 ± 2 °C, 45% relative humidity and a 12:12 h light–dark period. Insects reaching the fourth instar after ingesting a single blood meal as third instars, and newly emerged females were isolated and starved for 21 days, before a blood meal was offered. Only those insects fed *ad libitum* were selected for RNAi experiments.

2.2. Identification and mRNA expression of the RpMet gene

The sequence of the *TcMet* (ABR25244) was used to identify the *R. prolixus* ortholog in the genome database (http://vectorbase.org), using the TBLASTN algorithm and the BLOSUM62 matrix. The structure of the gene was predicted (i.e. ORF, introns and exons) using the two annotated mRNA sequences of *RpMet* and the *R. prolixus* genome with the Augustus software (http://augustus.gobics.de/).

Total RNA was extracted from the whole body of 4th instar larvae (fed and unfed pooled), as well as from different tissues of adult females (fed and unfed pooled) using the RNeasy kit according to manufacturer's specifications (Qiagen). RNA was treated with RNAse-free DNAse Set (Qiagen), and first strand cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA). The expression of *RpMet* was analyzed using the three specific set of primers listed in Table 1 (the position of every set of primers is shown in Supplemental Fig. 1). The PCR products were sequenced at the Unidad de Genómica, Instituto de Biotecnología, CICVyA-CNIA-INTA, Argentina.

Table 1
List of primers used to amplify the three different fragment of the *RpMet*.

Target	Sense	Sequence
HQ122610	Fwd	5′-ACCAAGCCGAGAAACAGCGT-3′
	Rev	5′-TTGAAGCCGAGCCTGGAGGT-3′
109 bp of the exon 3 of the Rp-MET isoform 1 (JN416985)	Fwd	**5′-**CTAAGTCTACAAAGGAAGTCAC-3′
	Rev	**5′-**CCTGACGAAGTGACAATC-3′
113 bp of the exon 4 of the Rp-MET isoform 2 (HQ122610)	Fwd	**5′-**TTACAGATTGGAAGAAACC-3′
	Rev	**5′**ATCTTCCCTGACGAAGTG-3′

2.3. Comparative analysis of the sequences

The following sequences from holometabolous species were utilized: ABR25244 (*Tribolium castaneum*), ACJ04052 (*Bombyx mori*), EHJ75902 (*Danaus plexippus*), AHX26585 (*Helicoverpa armigera*), AAF48071 (*D. melanogaster*), AFQ01087 (*Glossina morsitans morsitans*), AAX55681 (*Aedes aegypti*), AAY25027 (*Culex pipiens pipiens*), ABC18327 (*Anopheles gambiae*). The corresponding accession numbers for hemimetabolous species are: CDO33887 (*Blattella germanica*), AIM47235 (*Diploptera punctata*), AEW22976 (*Pyrrhocoris apterus*), the two predicted isoforms of the RpMet protein, and the ametabolous species *Thermobia domestica* (AEW22978). The sequence of the Met ortholog BAM83853 corresponding to *Daphnia pulex* (Crustacea: Brachiopoda) was also included. The functional domains of the protein encoded by *RpMet* were identified using the SMART software (Letunic et al., 2009). The similarity of the RpMet protein domains with other orthologs was determined by the multiple alignment ClustalW algorithm implemented in the CLC software. The analysis of the probable evolutionary relationships between sequences was performed using the maximum likelihood method based on the Poisson correction model, including a 1000 replicates bootstrap analysis, by the use of Mega 6.06 software (Tamura et al., 2013).

2.4. Gene expression knockdown

The dsRNA probe sequence was amplified by PCR using the second set of primers listed in Table 1, and corresponds to the fragment of isoform 1 that contains the third exon (Supplemental Fig. 1). The resulting amplicons were used as template in PCR reactions with primers containing T7 promoter sequences. The products from these PCR reactions were used to synthesize dsRNA with the Megascript RNAi kit (Ambion, Austin, TX) according to the manufacturer's recommendations. A total of 37 4th instar larvae and 14 newly emerged virgin females were intra-abdominal (ventral) injected with 0.4 μg (4th instar larvae), and 0.8 μg (adults) of *RpMet* dsRNA 72 h before feeding. Two additional groups of insects (4th instar larvae: 38; females: 12) were injected with a similar dose of yellow fluorescence protein (YFP) dsRNA (control group). After a blood meal was offered, only those insects fed *ad libitum* were selected.

2.5. Validation of RpMet mRNA silencing by RT-qPCR

Total RNA was extracted from pooled ovaries of insects injected either with control or *RpMet* dsRNA using the RNeasy mini kit (QIAGEN, USA) following manufacturer's instructions. Three biological replicates were used for each condition. An on-column treatment with RNase-free DNase I (QIAGEN, USA) was performed during RNA extraction to eliminate DNA contamination. Total RNA was quantified on a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA); the A_{260}/A_{280} ratio was used to check for protein

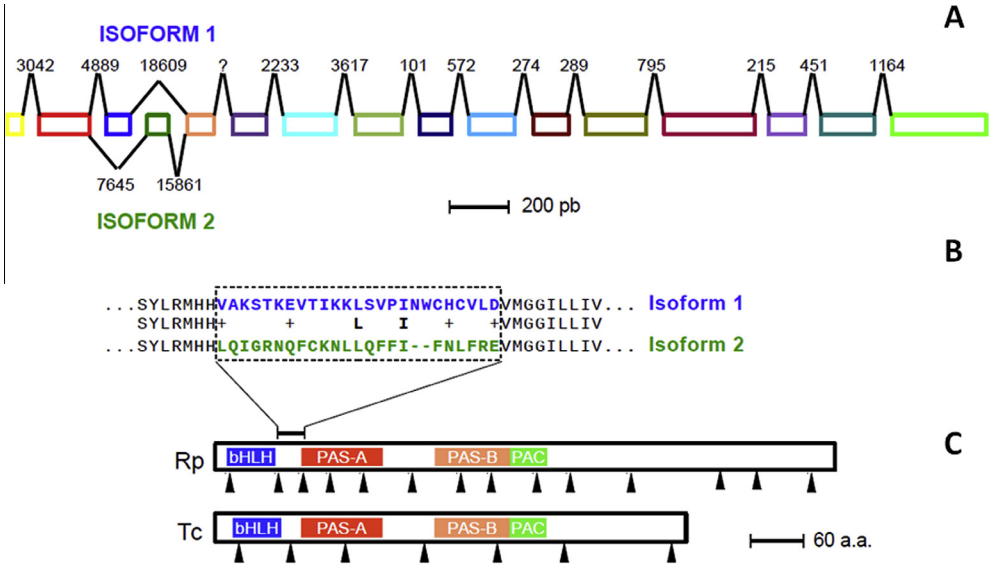

Fig. 1. Gene model and critical protein motifs of *RpMet*. (A) Organization of the *RpMet* gene showing the 16 exons (colored boxes). The numbers between exons indicate the size of each intron, while the color of the boxes correlates with the color of the corresponding exon sequences showed in Supplemental Fig. 1. Third and fourth exon (blue and green boxed) are mutually exclusive, generating the isoform 1 (mRNA: JN416985, protein: AEW22977) and isoform 2 (mRNA: HQ122610, protein: ADM47440) with 15 exons each. (B) Detailed comparison of a fragment between the bHLH and PAS-A domains of the two annotated isoforms showing the differences in the sequences of the mutually excluded exons. (C) Comparison of the conserved domains distribution along the complete Met proteins of *R. prolixus* and *T. castaneum*, black arrows indicate exon-exon junctions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

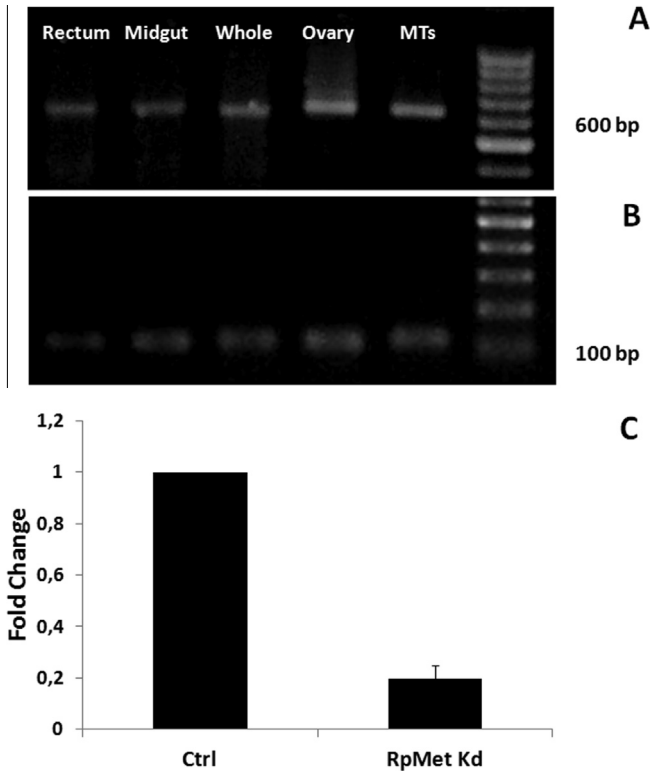

Fig. 2. Expression of *RpMet* in normal and RNAi-treated insects. (A) and (B) gels showing the expression of the two different *RpMet* fragments corresponding to the two distinct isoforms in several female organs (including ovary) and 4th instar larvae. The 567 bp fragment plus T7 promoter of the isoform 2 including exon number 4 (A) and the 109 bp fragment plus T7 promoter corresponding to the exon number tree expressed in isoform 1 (B). (C) Relative expression of *RpMet* in the ovaries of RNAi-treated *R. prolixus* females compared to the expression in the control group receiving YFP dsRNA. The control group was set as the reference (1-fold change).

contamination. An electrophoresis on a 1% denatured agarose gel stained with ethidium bromide was used to check RNA integrity and DNA residual contamination. First-strand cDNA was synthesized from 1 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad, USA), in 20 μl reaction volumes, following manufacturer's protocol. Temperature programming for reverse transcription was 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 30 min. RT-qPCR assay was performed on a Stratagene Mx3000P thermocycler (Agilent, USA) using the SYBR Green PCR Master Mix (Life Technologies, USA) according to manufacturer's protocols, in 20 μl reaction volumes containing a 10-fold dilution of cDNA and 200 nM of each primer. Results were normalized using *β-actin* and ribosome *S18* as reference genes (see Supplemental Fig. 3) (Majerowicz et al., 2011). Primer sequences for RT-qPCR analysis were: β-*actin* fwd, 5′-CACCCCAGCAATGTATGTAG-3′; *β-actin* rev, 5′-ACCATCAGGAAGTTCGTAAG-3′; *S18* fwd, 5′-TCCTTCGTGCTAGG AATTGG-3′; *S18* rev, 5′-GTACAAAGGGCAGGGACGTA-3′; 109 bp of the exon 3 of the *Rp-Met* isoform 1 (JN416985) (Table 1). The expression stability of the reference genes was checked using the NormFinder software (Andersen et al., 2004). Cycling conditions were: one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 3 min, 55 °C for 40 s, 72 °C for 30 s. A post-amplification melting curve analysis (one cycle at 95 °C for 1 min, 55 °C for 30 s, 95 °C for 40 s) was performed to confirm that a single product was being amplified (Supplemental Fig. 3). Two technical replicates were analyzed for each biological replicate in order to check for pipetting errors. No template and RT-minus controls were run to detect contamination, primer dimer formation and presence of genomic DNA. The products were run out on agarose gels to confirm their predicted amplicon size. PCR efficiency was assessed for reference and target genes by generating standard curves corresponding to a 5-fold dilution series (1:1 down to 1:625) from pooled cDNA (Supplemental Fig. 3). Gene expression levels, along with their standard errors, were calculated using the REST 2009 v2.0.13 analysis software.

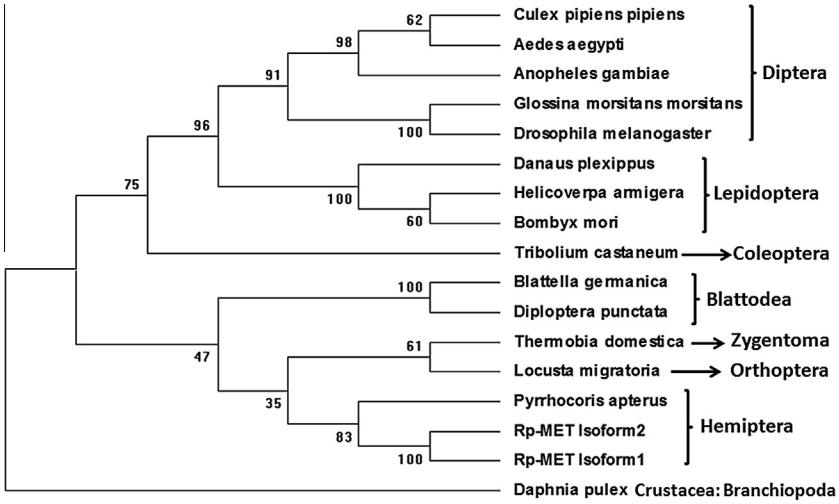

Fig. 3. Phylogenetic analysis of *Met* from different insect species. Phylogram showing the relationships between 16 insect species including one ametabolous, 4 hemimetabolous, 8 holometabolous and one crustacean (*D. pulex*) are based on the primary amino acid sequences as established by the Maximum Likelihood method based on the Poisson correction model. The tree represents the consensus after 1000 replicates bootstrap. Numbers on the branches indicate bootstrap percentage after 1000 replications in constructing the tree.

3. Results

3.1. Gene structure of RpMet

The analysis of the genomic contigs 17778.8 and 15877.1 (accession numbers: ACPB02032103, ACPB02020961) of the *R. prolixus* genome showed the presence of a region encoding a *Met* ortholog. The predicted gene model revealed the presence of 16 exons (Fig. 1A). As in *T. castaneum*, the deduced protein contains one basic Helix-Loop-Helix domain (bHLH), two Pert-Arnt-Sim domains (A and B) (PAS), and a PAS C-terminal motif PAC (Fig. 1C).

3.2. RpMet expression

By the use of specific primers (first set of primers in Table 1) we obtained a 567 bp fragment of the *RpMet* transcript encoding a fragment of the protein spanning 189 amino acids. This fragment was annotated with the accession numbers (mRNA: HQ122610, protein: ADM47440).

The *RpMet* transcript was amplified using as template cDNA obtained from different adult organs (hindgut/rectum, whole midgut, ovary, Malpighian tubules), and also from cDNA obtained from a pool of tissues of fed and unfed 4th instar larvae (Fig. 2A).

Another *RpMet* transcript fragment of 2133 bp was annotated with accession numbers (mRNA: JN416985, protein: AEW22977) (Konopova et al., 2011). The comparison of both annotated sequences reveals that differ in a fragment of 75 bp, suggesting the existence of an alternative splice variant in the *RpMet* gene (Fig. 1B). In fact, based on the 16 exons predicted and both annotated sequences, the third and fourth exons are expressed in a mutually exclusive way in two isoforms (RpMet isoform 1 and RpMet isoform 2) (Fig. 1B, Supplemental Fig. 1). The mutually exclusive exons correspond to the protein region that connects the two conserved domains bHLH and PAS-A (Fig. 1B and C).

To attempt a differential functional analysis of these two variants, we designed two additional sets of primers (second and third pairs of primers in Table 1). Each set of primers was intended to amplify the specific fragment corresponding to each one of the mutually exclusive exons. The third pair of primers (Table 1) did not generate amplicons with any of our cDNA sources used as template. Using the second set of primers we were able to obtain a 109 bp fragment in all the cDNA analyzed (Fig. 2B).

3.3. Comparative analysis of the sequences

The sequences of the predicted RpMet proteins were aligned with related proteins corresponding to holometabolous and hemimetabolous insects, as well as the ametabolous species *T. domestica* and the Crustacea *D. pulex*. Functional protein motifs (indicated with different color bars in the alignment) were well conserved in all the sequences (Supplemental Fig. 2). The hemimetabolous species (i.e. *R. prolixus*, *P. apterus*, *Locusta migratoria*, *B. germanica* and *D. punctata*), appeared closely related to the ametabolous species *T. domestica*, while the holometabolous species were grouped in a separate cluster, with a distribution that was consistent with the taxonomy established for the corresponding orders. The sequence corresponding to *D. pulex*, the only non-insect species included in the analysis, acted as the out-group rooting the whole cluster of insect species (Fig. 3).

3.4. Functional analysis

Fourth-instar larvae were treated with *RpMet* or control dsRNA. After 72 h, a meal was offered to induce the molt to the pre-metamorphic fifth instar. Only those insects fed *ad libitum* were selected ($n = 28$). After molting, 78.6% of the individuals treated with *RpMet* dsRNA showed phenotypic alterations; presenting a mix of juvenile and adult characters (adultoids), which included wings not properly developed and morphological alterations in the genitalia (Fig. 4A–F). All the insects treated with control dsRNA ($n = 20$) underwent normal molts to fifth instar. Most of the control insects reached the adult stage normally, while most of the adultoids were not able to get further meals. A number of adultoids that reached a new meal were not able to molt and died.

Newly emerged virgin adult females were also injected with *RpMet* or control dsRNA 72 h before receiving their first adult meal. All the insects in the control group developed normal ovaries (Fig. 5A, C and F), while 71.43% of the females injected with *RpMet* dsRNA showed no ovary development (Fig. 5B). The remaining females displayed different degrees of ovary development, and in some cases even laid eggs. The size and the color of those eggs were abnormal, suggesting that vitellogenesis failed. Actually, the diameter of the operculum, as well as the total length and perimeter, were lower in those eggs laid by the females treated with *RpMet* dsRNA (Fig. 6). Regarding the color, all eggs (laid or not)

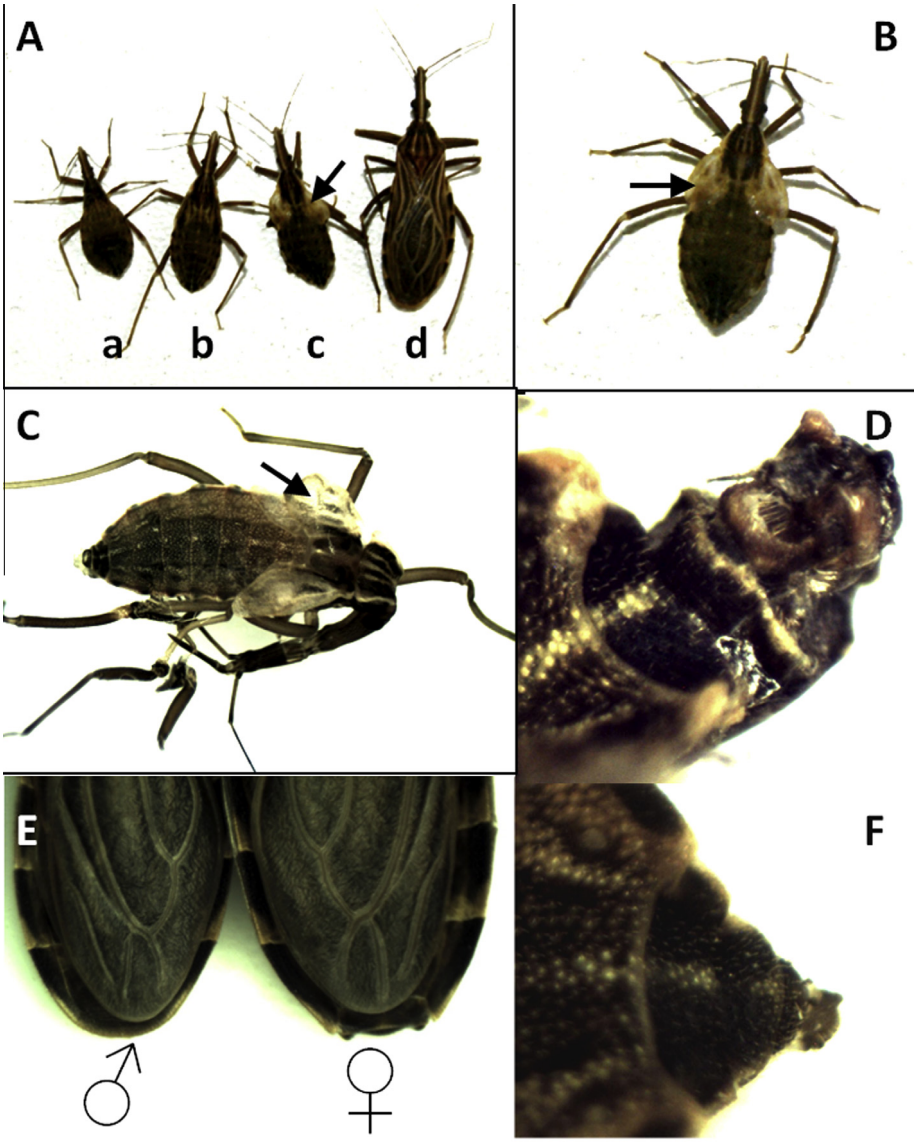

Fig. 4. Phenotypic alterations caused by the *RpMet* interference in *R. prolixus* larvae. (A) General view comparing a normal fourth-instar larva (a), with a normal fifth-instar larva obtained after treatment with YFP dsRNA (control) (b), a normal adult (d) and a resulting adultoid after treatment with *RpMet* dsRNA (c). (B) General appearance of another adultoid obtained after molting of fourth-instar larvae injected with dsRNA (the abnormal growth of the wings is evident – arrow). (C) General appearance after manual removal of the cuticle of an adultoid that was fed again and died during the next moult (the arrow shows the wings with abnormal development). (D) Detailed dorsal view of the aspect of the altered morphology of the genitalia of an adultoid. (E) and (F) Dorsal views of the developed genitalia in a normal male and female (E) and in a fifth instar larva (F).

did not present the characteristic pink color, caused by the accumulation of the Rhodnius heme-binding protein (RHBP) (Fig. 5D –F).

The expression of the *RpMet* mRNA in ovaries from control and treated females was quantitatively analyzed by RT-qPCR. The average threshold cycle (Ct) of the treated samples was 22.73 ± 0.47, while the average Ct of the control samples was 20.66 ± 0.24 (Supplemental Fig. 3), showing that the expression of *RpMet* was reduced by 80.3% in the ovaries from treated females, when compared to ovaries from control ones (Fig. 2C).

4. Discussion

Juvenile hormones are key factors in insect development and reproduction. Understanding the mode of action of JHs at the molecular level has been a major challenge in insect biology. The recent discovery that the JH-resistance gene, *Methoprene-tolerant* (*Met*), plays a critical role in insect metamorphosis has been followed by a rapid increase in our understanding of JH signaling. In the absence of JH, Met is present as an inactive homodimer. Upon JH binding to the PAS-B domain, Met undergoes a conformational change that liberates it from the homodimer complex and allows it to bind a new partner. In the mosquito *A. aegypti*, in the presence of JH, Met forms a dimer with FISC (also called TAIMAN), another member of the bHLH-PAS family of genes (Li et al., 2011). In fact, the expression of several JH-responsive genes is significantly altered in newly emerged female mosquitoes after the suppression of the activity of any of the two bHLH-PAS genes (Li et al., 2011). In *T. castaneum* (Minakuchi et al., 2009) and *R. prolixus* (Konopova et al., 2011), the anti-metamorphic effects of *Met* are mediated by genes acting downstream, such as the *Krüppel homolog 1* (*Kr-h1*); this transcription factor is also involved in the JH-dependent maturation of sexual behavior in males of the moth *Agrotis ipsilon* (Duportets et al., 2012).

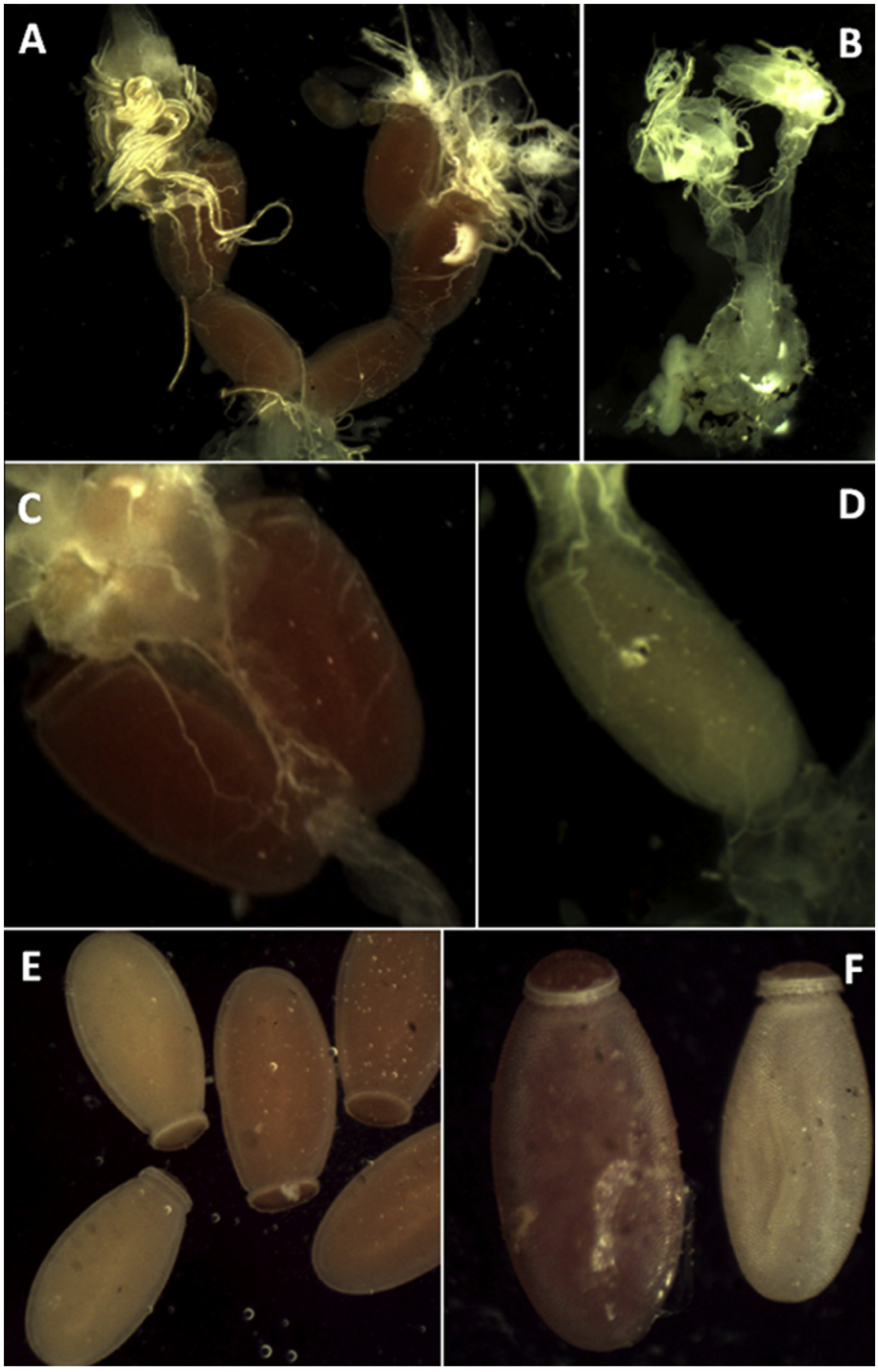

Fig. 5. Ovary alterations after *RpMet* interference. (A) General view of the reproductive system of a control treated female. (B) General view of the ovary of a female treated with *RpMet* dsRNA. C and D: Aspect of developing oocytes in a control female (C) and a female treated with *RpMet* dsRNA (D). Note that the difference of the overall color of the oocytes is evident showing the lacking of accumulation of RHBP in silenced females (D). (E) several deposited oocytes by females undergoing *RpMet* silencing showing different degrees of pink colorless. F: Comparison between two eggs laid by a control female (left) and *RpMet* silenced female (right), showing the difference in color and size.

Met was originally characterized in *D. melanogaster* (Ashok et al., 1998); but studies in *T. castaneum* provided the first evidence that the encoded protein acts as a putative JH receptor associated with the control of metamorphosis in holometabolous insects (Konopova and Jindra, 2007). More recently its anti-metamorphic role was also verified in the hemimetabolous species *P. apterus* and *B. germanica* (Lozano and Belles, 2014; Konopova et al., 2011). Previous studies in *R. prolixus* and *Triatoma infestans* revealed that chemical allatectomy using Precocene II before or during the first days after blood-meal led to alterations in the metamorphic process, generating a classical "adultoid" phenotype with a mix of juvenile and adult characters (Ronderos, 2009; Stoka et al., 1988; Tarrant and Cupp, 1978). A similar adultoid phenotype was obtained after *RpMet* mRNA depletion, advocating that *RpMet* is involved in the control of metamorphosis in Triatominae.

JHs also play an important role in the adult female regulating the synthesis and incorporation of vitellogenins in several insect species. In fact, it was recently shown that *Met* knockdown inhibits

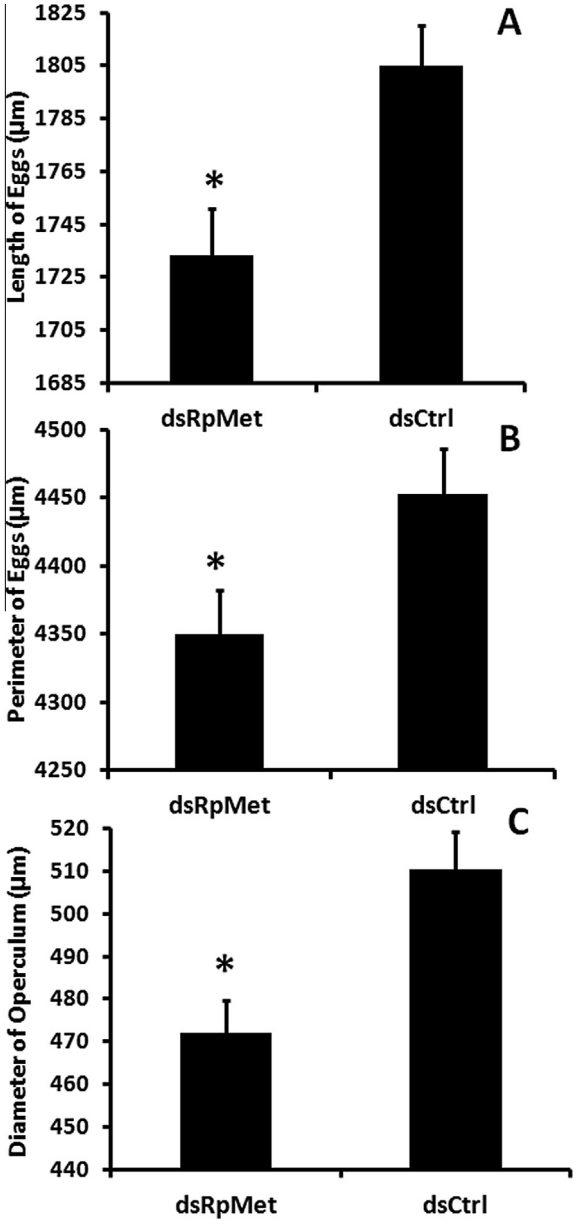

Fig. 6. Morphological analysis of laid eggs by females undergoing normal and altered development. (A) Differences at the level of the length of the oocytes. (B) Differences between the overall perimeter of the egg in normal and silenced females. (C) Differences observed at the diameter of the operculum. Statistical differences were established by ANOVA. Bars represent media ± S.E. of 30 eggs deposited by control ($n = 30$) and *RpMet* dsRNA treated females ($n = 31$).

ovarian development in the cockroach *D. punctata* (Marchal et al., 2014). Our functional studies also confirmed the importance of *RpMet* for the normal reproductive maturation of adult females. The silencing of *RpMet* caused a significant alteration on vitellogenesis, a process known to be under JH control in Triatominae insects (Coles, 1965; Wang and Davey, 1993). This was evidenced not only by a significant decrease in ovary development, but also by the alteration of the size and color of the developing eggs. The characteristic pink coloration of *R. prolixus* eggs is due to the presence of a hemo-binding protein. *R. prolixus* ingests great quantities of hemoglobin with the blood meal. Digestion of hemoglobin releases heme that binds to a hemo-binding protein to avoid oxidative damage (Dansa-Petreski et al., 1995). This protein, named RHBP was first characterized by Oliveira et al. (1995), but the presence of a "colored" protein entering into the oocytes was first described by Wigglesworth (1943). RHBP is necessary for the normal development of the oocytes (Machado et al., 1998). Our results clearly show that the normal incorporation of RHBP in the eggs was altered after RpMet depletion, suggesting that, as other *R. prolixus* yolk proteins, RHBP synthesis and/or incorporation is controlled by JH.

Interestingly, we have also detected the expression of *RpMet* in the rectum, salivary glands and Malpighian tubules, which are not typically considered as targets of JH; the presence of Met and the effects of JH in those tissues has been less investigated (Pursley et al., 2000). Although, an activity of JH on these organs in the Colorado potato-beetle *Leptinotarsa decemlineata* has been previously reported (Yi and Adams, 2001).

The existence of two *RpMet* isoforms can be predicted from the *R. prolixus* genome. Indeed two sequences differing in one exon (located between bHLH and PAS-A domains) have been annotated for *RpMet*, showing the existence of a mutually exclusive splicing mechanism for the corresponding protein. The existence of *Met* isoforms might be a common fact. Indeed the existence of two different isoforms was confirmed for *TcMet* (XM_008193217 and NM_001099342). Unfortunately, despite of the efforts made to perform a functional assay of each isoform independently, only one of them could be analyzed by RNAi experiments under our experimental conditions.

In summary, these studies validated that RpMet is a member of bHLH-PAS family of proteins showing the presence of all critical active domains. The striking results of the reverse genetic experiments showing precocious metamorphosis in larvae and alterations in the vitellogenetic processes in adult females, strongly suggest that RpMet is involved in the complex of signals by which JHs exert its activity in *R. prolixus*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2015.04.018.

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