A sex-specific homologue of snake *waprin* is required for embryonic development in the Red Flour Beetle, *Tribolium castaneum*.

Chhavi Choudhary, Saloni Sharma, Keshav Kumar Meghwanshi, Divyanshu Kishore, Vivek Verma, Jayendra Nath Shukla*

#Department of Biotechnology, School of Life Sciences, Central University of Rajasthan, Ajmer, 305817 Rajasthan, India.

***Corresponding author, email ID: jayendrashukla@curaj.ac.in**

1. Abstract:

Waprin, first discovered in snake venom, is an extracellular secretory protein known for its antibacterial activity. Analysis of sex-specific transcriptomes of adult *Tribolium castaneum* led to the identification of a female-specific Waprin protein gene (Tc_Wap^F) which expresses only in female adults. Knockdown of Tc_Wap^F didn't show any effect in the same generation, but the eggs laid by Tc_Wap^F knockdown females failed to develop/hatch. No expression of Tc_Wap^F was detected in *Tribolium* embryos, hence considering the antibacterial properties of Waprin proteins and the failure of hatching of eggs laid by Tc_Wap^F knockdown females, we assume the involvement of Tc_Wap^F in protecting the embryos from the environmental pathogens. Tc_Wap^F is not regulated by sex determination pathway of *T. castaneum* as the expression of Tc_Wap^F was not changed upon the knockdown of sex determination pathway genes of *T. castaneum*. Further, we also identified two more *waprin* genes of *T. castaneum* expressing in a non-sex specific manner at all the developmental stages. To the best of our knowledge this study is the first attempt to characterize the developmental role of *waprin* genes in any insect. The study presented here provides an opportunity to probe the potential of *Tribolium* Waprin proteins as non-conventional antimicrobials.

2. Introduction:

Waprin, a WAP (Whey Acidic Protein) domain containing secretory protein, was first identified in the venom of Black-necked spitting cobra, *Naja nigricollis*, and was named Nawaprin (Torres et al. 2003). Later studies demonstrated the ubiquitous nature of Waprin proteins across genera (Alam et al. 2019). Waprin proteins were primarily identified in the

extracellular secretions from mammals (whey protein in milk), snakes (St. Pierre et al. 2008; Reeks, Fry, and Alewood 2015), insects (Bouzid et al. 2014), scorpions (Romero-Gutierrez et al. 2017; Romero-Gutiérrez et al. 2018), cnidaria (Zhang et al. 2007) and frogs (Ali et al. 2002; Liu et al. 2013) etc. Waprin proteins discovered in the venoms of *N. nigricollis* (Torres et al. 2003), Inland taipan snake (*Oxyuranus microlepidotus*) (Nair et al. 2007) and Venezuelan horned frog (*Ceratophrys calcarata*) (Liu et al. 2013) has been structurally and functionally characterized. Besides, the presence of *waprin* like gene has been reported in the transcriptome of several other organisms like scorpions (Romero-Gutierrez et al. 2017; Romero-Gutiérrez et al. 2018), cnidarians (Klompen et al. 2020) and ants (Bouzid et al. 2014). The peculiar feature of Waprin protein is the presence of eight cysteine residues at the core of the protein, which are involved in the formation of four disulphide bonds (Suntravat et al. 2016; Alam et al. 2019).

Interestingly, the WAP domain proteins have been reported to have multifarious activities including antiviral activity (Chen, He, and Xu 2008), antimicrobial activity (Ali et al. 2002; Hagiwara et al. 2003; Hauton, Brockton, and Smith 2006; Smith et al. 2008; Imjongjirak et al. 2009; Li et al. 2013), Na-K ATPase inhibitory activity, protein and proteinase inhibitory activities (Ali et al. 2002; Li et al. 2013), as well as immunomodulatory functions (Gao et al. 2021). Antimicrobial activity against various gram-positive and gram-negative bacteria and antifungal activity against certain fungi are the most widely studied functions of Waprin proteins (Liu et al. 2013). For instance, ceratoxin from the skin secretions of *C. calcarata*, has been shown to inhibit *Staphylococcus aureus*, a gram-positive bacterium (Liu et al. 2013) whereas Waprin protein (Omwaprin) of *O. microlepidotus* works selectively against the strains of *Staphylococcus warneri* and *Bacillus megaterium*, gram negative bacteria (Nair et al. 2007). The antimicrobial activity of Omwaprin is salt tolerant up to 350mM NaCl and this activity is lost upon reduction and alkylation of its eight cysteine residues or upon disruption of its six N-terminal residues, four of which are positively charged. This suggested that the four disulphide bonds are crucial for the antibacterial activity of Waprin proteins (Nair et al. 2007). In a recent study, Thankappan et al (2019) used Omwaprin to derive two cationic antimicrobial peptides (Omw1 and Omw2) with enhanced antimicrobial, antifungal, and antibiofilm activities (Thankappan and Angayarkanni 2019).

Identification of transcripts related to *waprin* is limited only to a few insect species i.e., in the wood-boring woodwasp, *Sirex nitobei* (Gao et al. 2021) and in guinea ant, *Tetramorium bicarinatum* (Bouzid et al. 2014), but the first ever study to show the antibacterial potential of

any insect Waprin has recently been done for the Waprin (Amwaprin) of honeybees (*Apis millefera*). Recombinant Amwaprin produced in baculovirus-infected Sf9 cells was shown to exhibit microbicidal and anti-elastolytic activities (Lee et al. 2023). The function of *waprin* in *T. bicarinatum* is not known but the *waprin* of *S. nitobei* has been hypothesized to promote the growth of a symbiotic fungus, *Amylosterium areolatum*. During oviposition, *S. nitobei* female injects its venom along with arthrospores of *A. areolatum* into the tree trunk. *A. areolatum* helps in the growth of *S. nitobei* eggs and larvae by providing nutrition to them and in return, Waprin present in the venom of *S. nitobei* inhibits the bacterial growth, inturn facilitating the growth of *A. areolatum* (Coutts 1969b; 1969a; Gao et al. 2021).

The red flour beetle, *Tribolium castaneum*, is a pest of stored food grains. Its wide geographical range and infestation potential in a variety of food products makes it an economically important pest. Besides, the extent of RNAi efficiency in *T. castaneum* makes it an excellent model for reverse genetics studies (Campbell et al. 2021). Screening of sex-specific transcriptomes (unpublished) of six-day old adult *T. castaneum* led to the identification of a female-biased (FPKM value-179.20 and 0.35 for females and males respectively) transcript of *waprin* (Tc_Wap^F) gene. qRT-PCR using sex-specific (female and male adults) *Tribolium* cDNAs and Tc_Wap^F specific-primers confirmed the female-exclusive expression of Tc_Wap^F. Developmental and tissue-specific profiling suggests the expression of Tc_Wap^F exclusively in all the tissues of female adults. RNAi mediated knockdown in the expression of Tc_Wap^F in adult females did not produce any phenotypic alteration in the injected individuals whereas its parental RNAi resulted in the failure of hatching of eggs laid by Tc_Wap^F knockdown females. Since, Tc_Wap^F is not expressed in embryos and parental RNAi of Tc_Wap^F leads to the failure in the hatching of eggs, we hypothesize that Tc_Wap^F is maternally supplied for the development of embryos. Considering the antimicrobial potential of Waprin proteins (Amwaprin in particular) (Lee et al. 2023), we further hypothesize that the maternal transfer of Tc_Wap^F has evolved to provide antimicrobial properties to the embryos. Sex-specific expression and identification of a consensus Doublesex (Dsx), the most downstream gene of insect sex-determination cascade binding site in the upstream region of Tc_Wap^F prompted us to explore whether the expression of Tc_Wap^F is controlled by the sex determination pathway genes of *T. castaneum*. For this we performed the RNAi mediated knockdown of *T. castaneum* transformer (*Tctra*) and *dsx* (*Tcdsx*) genes and compared the expression of Tc_Wap^F between control (ds_GFP injected) and knockdown (*dsTctra* or *dsTcdsx* injected) individuals (Shukla and Palli 2012a; 2012b; 2013; 2014). Surprisingly, the

expression of *Tc_Wap*^{F} was unaltered upon the knockdown of *Tctra* or *Tcdsx* suggesting *Tcdsx* and *Tctra* doesn't have any role in regulating the female-specific expression of *Tc_Wap*^{F}. We identified two more *waprin* genes (*Tc_Wap*^{2} and *Tc_Wap*^{3}) of *T. castaneum*; however, they were found to express constitutively at all the developmental stages and in both the sexes. To the best of our knowledge this study is the first to identify and characterize the biological significance of a sex-specific insect *waprin* gene. Besides this is the first report of presence of multiple *waprin* genes in any insect.

3. Materials and methods:

3.1 *Tribolium castaneum* strain and rearing:

Wild type strain of *T. castaneum* was reared on whole-wheat flour supplemented with 5% yeast in an incubator set at 30°C temperature and 60-70% relative humidity. Sexing of pupae and adults were done by observing the presence or absence of sexually dimorphic characters (Shukla and Palli 2012b) with the help of Leica s9i stereomicroscope (Leica microsystems, Germany).

3.2 RNA Isolation and RT-PCR:

Total RNA from different developmental stages of *Tribolium* eggs, 6th instar larvae, Q stage, sex-specific pupae, sex-specific adults and dissected tissues (gut, ovary, head and fatbody) from female adults were isolated using Trizol reagent (Invitrogen, USA) and were DNAse (Promega, USA) treated. The concentrations of DNA free RNA samples were taken using NanoDrop and the RNA samples were stored at -20°C, until its further use. RNA samples (3µg) were incubated at 70°C for 5 min and were immediately chilled on ice. First strand cDNA was synthesized using GoScript Reverse Transcriptase (Promega, USA) using 18-mers polyT primer, according to the manufacturer's instructions. The prepared cDNA was diluted 10 folds with nuclease free water and used for subsequent PCR studies. GoTaq polymerase (Promega, USA) was used for all the RT-PCR reactions using gene-specific primers (Supplementary Table-1) which were designed using Primer-3 software (Primer3 Input (version 0.4.0). PCR conditions were as follows- Initial denaturation at 94°C for 2 min, 32 cycles of 94°C for 30 s, annealing temperature (supplementary table 1) 30 s, 72°C for 2 min and the final extension at 72°C for 10 min. The amplified products were electrophoresed on 1.5% agarose gels and the gels were scanned in a gel documentation system (BioRad, USA).

3.3 Sequence analysis:

After identification of *Tc_Wap*^{F} gene from our transcriptome data (unpublished), its sequence was confirmed using BLAST (BLASTn) searches in the EST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_SPEC=GDH_GCF_000002335.3) of *T. castaneum*. Sequences of other *waprin* genes (*Tc_Wap*^{2} and *Tc_Wap*^{3}) of *T. castaneum* were obtained by tBLASTn searches in the EST database of *T. castaneum* using Tc_Wap^F protein as a query sequence. Exons and introns of all the *Tc_waprin* genes (*Tc_Wap*^{F}, *Tc_Wap*^{2} and *Tc_Wap*^{3}) were identified by aligning the gene-specific EST sequences with their corresponding genomic DNA sequences using Splign software (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi).

3.4 Double stranded RNA (dsRNA) synthesis and injections:

cDNA from adult females and gene-specific primers (supplementary Table-1) containing the T7 promoter sequences at their 5' ends were used to PCR amplify the specific regions of *Tc_Wap*^{F}, *Tcdsx* and *Tctra* genes. Purified amplicons were used as template for in-vitro transcription reaction using T7 RiboMAX™ Express RNAi System (Promega, USA) according to the manufacturer's instructions. The control dsRNA was made using an amplified fragment of the GFP gene sequence.

Newly emerged (0 day) female adults (5 h PAE) were immobilized by keeping them at 4°C for 8–10 minutes prior to injections. Unconscious adults were injected with dsGFP (control) or with dsRNA specific to one of the genes (*Tc_Wap*^{F}, *Tcdsx* or *Tctra*) using an aspirator tube assembly (Sigma) attached to a 3.50 glass capillary tube (Drummond Scientific) drawn by a micropipette puller (Narishige, PC-100). Approximately, 500–600 ng dsRNA was injected per insect. Injected insects were starved and given time to recover at room temperature for around 5 to 6 hours before transferring them to the incubator under normal rearing conditions. Knockdown efficiency of *Tc_Wap*^{F} and *Tcdsx* were analyzed after 5 days of injections whereas knockdown efficiency of *Tctra* and its effect on *Tc_Wap*^{F} was analyzed on 3 days and 5 days after injections. The ratio of gene expression between beetles injected with target dsRNA and those injected with GFP dsRNA was used to calculate the efficacy of gene knockdown in the RNAi insects.

3.5 Parental RNAi:

To see the effect of *Tc_Wap*^{F} knockdown in the next generation, control or gene specific dsRNA was injected to virgin adult females (0 day) as described in the previous section (3.4). Injected females were allowed to mate with their counterparts (uninjected males of the same age) for 6 days; one mating pair per cup was maintained. All the adults were removed from the cups and females (*Tc_Wap*^{F} or dsGFP injected) were dissected to compare their tissue morphology. Isolated tissues were examined under microscope and their images were captured. The number of eggs in each cup was counted, the development of eggs was monitored and eggs were photographed periodically.

3.6 qRT-PCR Analysis:

Total RNA isolation from control and knockdown females, and first strand cDNA synthesis was carried out as described in section 3.2 Synthesized cDNAs and gene-specific primers (Supplementary Table 1) were used in qRT-PCR reactions to determine the relative mRNA levels of *Tc_wap* genes using GoTaq® qPCR Master Mix (Promega) according to the manufacturer's instructions. Three independent biological replicates were used for each treatment. *T. castaneum* ribosomal protein (RPS-18) gene was used as an endogenous control to normalize the expression data (Toutges et al. 2010), and 2-ΔΔCT method was employed to assess the gene expression levels (Livak and Schmittgen 2001). The qPCR reactions were carried out in a CFX96 touch real-time PCR detection system (Bio-Rad, USA) with following cycling parameters: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 45 s and 72 °C for 5s. Melt curve analysis was carried out at 60–95°C, in steps of 0.5 °C increments per 5s. The software GraphPad Prism (9.5.1) was used to analyze statistical data. Two tailed t-test and one way ANOVA were used to compare the gene expression between different samples.

3.7 Phylogenetic Analysis and Domain Architecture:

Phylogenetic analysis of Waprin proteins of different organisms was performed using the iTOL tool which recognizes the Newick file. Predicted protein sequence of *T. castaneum*

Waprin (XP_008198656.1) was retrieved from NCBI and BLAST (blast-p) analysis was performed in the non-redundant (nr) protein database. The obtained protein sequences (FASTA) were aligned using MUSCLE and were further used to construct the phylogenetic tree using the Maximum likelihood method. The FASTA sequences of Tc_Wap^2, Tc_Wap^3, Nawaprin and Omwaprin (1,11) were also included in the analysis. Domain searches in the Waprin proteins of different organism were conducted using Conserved domain database (https://www.ncbi.nlm.nih.gov/cdd/) and motif scan (https://myhits.sib.swiss/cgi-bin/motif_scan), an online software. Eight conserved cysteine residues were used as a signature to identify the conserved domains and establish the relationship between their associations and divergence.

4. Results:

4.1. RNA Isolation, cDNA synthesis and RT-PCR:

Transcripts of *waprin* were analyzed by semiquantitative RT-PCR at all developmental stages (eggs, 6th instar larva, Q stage larva, female pupa, male pupa, female pupa, female adult, and male adult) of *Tribolium* using gene-specific primers. *Tc_Wap^F* was found to express exclusively in adult females whereas *Tc_Wap^2* and *Tc_Wap^3* express at all the developmental stages of *Tribolium* (Figure 1A). In addition, *Tc_Wap^F* transcripts were detected in all tissues of females, including the ovary, gut, head, and fatbody (Figure 1B). Interestingly, expression of *Tc_Wap^F* was not observed (a faint band at 40hrs egg stage was not reproduced) at any of the egg stages whereas the expression of other two *waprin* genes (*Tc_Wap^2* and *Tc_Wap^3*) gradually increased with the embryonic development (Figure 1C). Consistent with the result of semi-quantitative PCR, we observed approximately 700-fold high expression of *Tc_Wap^F* in females compared to males in qRT-PCR analysis (Figure 1D).

4.2 Gene structure and sequence analysis:

The mRNA of *Tc_Wap^F* (XM_008200434.2) is 573 nucleotides long and harbors 2 exons and 1 intron. The ORF of *Tc_Wap^F* is 240 bases encoding 80 amino acids (aa) polypeptide. The mRNAs of *Tc_Wap^2* (XM_964663.4) and *Tc_Wap^3* (XM_964731.3) are of 578 and 512 bases respectively. *Tc_Wap^2* contains 3 exons and 2 introns while *Tc_Wap^3* contains 2 exons and 1 intron and their ORFs encode for 116 aa and 122 aa polypeptides respectively

(Supplementary figure 1). Eight conserved cysteine residues, a distinctive feature of the WAP domain, were identified in all three Waprin proteins of *T. castaneum*.

4.3. Knockdown of *Tc_Wap*^{F} and its Phenotype:

Knockdown of *Tc_Wap*^{F} was confirmed through semi-quantitative RT-PCR using cDNA of control (ds*GFP* injected) and knockdown females (ds *Tc_Wap*^{F} injected) (Supplementary figure 2). Further ~12-fold reduction in the expression of *Tc_Wap*^{F} in knockdown females as compared to control females was observed in qRT-PCR analysis (Figure 2A). We observed a slightly higher number of egg laying by *Tc_Wap*^{F} knockdown females as compared to that of control females (Figure 2B). However, the eggs from knockdown females failed to develop/hatch, whereas eggs from control females developed normally and eggs were hatched (within 65hrs) into larvae (Figure 2C and Supplementary figure 3).

4.4 Effect of knockdown of *Tcdsx* and *Tctra* genes on the expression of *Tc_Wap*^{F}:

A conserved *dsx*-binding motif (ACAATGT) was found in the upstream region (4.5 Kbp upstream of the *Tc_Wap*^{F} transcription start site) of *Tc_Wap*^{F}. This prompted us to investigate whether the sex-specific expression of *Tc_Wap*^{F} is regulated by the TcDsx transcription factor of *T. castaneum*. Injection of *Tcdsx* dsRNA (ds*Tcdsx*) lead to the significant knockdown in the expression of *Tcdsx* in both male and female adults (Figure 3A) but no change in the expression of *Tc_Wap*^{F} was observed in *Tcdsx* knockdown adults (females or males) as compared to control adults (females or males) (Figure 3B).

We further hypothesized that female-specific expression of *Tc_Wap*^{F} could be regulated by some other sex-specific transcription factor (other than TcDsx) controlled by *TcTra*, a splicing factor responsible for female specific splicing of *Tcdsx* pre-mRNA (Shukla and Palli 2012b). Hence, knockdown of *Tctra* was done by injecting the dsRNA of *Tctra* in female *Tribolium* adults and the expression of *Tc_Wap*^{F} was compared between control and *Tctra* knockdown females. Both female and male isoforms of *Tctra* were obtained in RT-PCR of *Tctra* Knockdown females (Supplementary figure 4), suggesting a successful knockdown of *Tctra* in the ds*Tctra* injected females (Shukla and Palli 2014). Surprisingly, there was no effect in the expression of *Tc_Wap*^{F} in *Tctra* knockdown females; neither at 3 nor at 5 days after ds*Tctra* injections (Figure 3C).

4.5 Phylogenetic Analysis and Domain Architecture:

A total of 65 Wap and Wap related protein sequences from seven different orders of class insecta and six outgroups (including bacteria, crustaceans, scorpions, reptiles, amphibians and mammals) were retrieved using the BLASTp tool. The phylogenetic tree generated using the Maximum likelihood method grouped the proteins into three clusters. The relationships between all three Waprin of *Tribolium* (Tc_Wap^{F}, Tc_Wap^{2}, Tc_Wap^{3}) was established; the three Wap diverged into different sister branches despite belonging to the same parent branch. Tc_Wap^{F} was closely related to waprin of *Tribolium madens* while *Sandaracinus amylolyticus* and Tc_Wap^{2} have similar protein sequences and were closely related. Similarly, Tc_Wap^{3} has a protein sequence similar to that of *Hermetia illucens*. Figure 4 depicts that Tc_Wap^{F} and Tc_Wap^{3} are distantly related to each other and Tc_Wap^{2} lies intermediate to both, Tc_Wap^{F} and Tc_Wap^{3} (Figure 4).

The hallmark of Waprin and Waprin-related proteins throughout the animal kingdom is the presence of eight conserved cysteine residues that produce four disulfide linkages along with the WAP domain (Alam et al. 2019; Suntravat et al. 2016). Domain architecture analysis using TB tools predicted the presence of WAP domains across protein sequences collected from seven different orders of insects. Analysis of the domain organization of all the predicted WAP proteins across major orders and representative species showed that the WAP domain is conserved across all members. WAP protein of few species were found to possess multiple WAP domains as well as additional domains, other than the WAP domain. Tc_Wap^{F} showed the presence of single WAP domain while Tc_Wap^{2} consists of two WAP domains. Although Tc_Wap^{3} contains eight conserved cysteine residues, this tool indicated the absence of the WAP domain in Tc_Wap^{3}. Kunitz domain, a serine protease inhibitor found in the venom of several animals including snakes, lizards, spiders, ticks, and sea anemones is the second most conserved domain across 65 species (Figure 5).

5. Discussion:

Waprin (WAP domain containing proteins) are found in diverse groups of organisms (Alam et al. 2019) and are known to possess antiviral (Chen, He, and Xu 2008) , antimicrobial(Ali et al. 2002; Hagiwara et al. 2003; Hauton, Brockton, and Smith 2006; Smith et al. 2008; Imjongjirak et al. 2009; Li et al. 2013), Na-K ATPase inhibitory, protein and proteinase inhibitory(Ali et al. 2002; Li et al. 2013), as well as immunomodulatory functions (Gao et al. 2021). Waprin protein has been reported from a few insect species also for e.g., wood-boring woodwasp, *S. nitobei* (Gao et al. 2021), guinea ant, *T. bicarinatum*(Bouzid et al. 2014) and

honeybees (*A. millefera*). Besides the microbicidal and anti-elastolytic activities of Amwaprin (Lee et al. 2023), a recently discovered Waprin protein from *A. melifera* not much is known about the biological significance and evolution of Waprin proteins in insect kingdom.

We identified three Waprin proteins from the red flour beetle, *T. castaneum*. Most importantly, one of the *Tribolium* Waprin proteins (Tc_Wap^{F}) was found to express only in adult females. Since Tc_Wap^{F} has the typical feature of Waprin protein i.e., the presence of eight conserved cysteine residues that produce four disulfide linkages along with the WAP domain (Alam et al. 2019; Suntravat et al. 2016) we assumed its functional conservation. To deduce the biological relevance of *Tc_Wap^{F}*, RNAi mediated knockdown of *Tc_Wap^{F}* was done in adult females. Despite the significant knockdown in the expression of *Tc_Wap^{F}* no observable change was noticed in the morphology or anatomy of the knockdown individuals. Considering the antiviral and antimicrobial activities of Waprin protein one can expect enhanced microbial infections in the *Tc_Wap^{F}* knockdown individuals but since our *Tribolium* cultures were maintained in a clean and optimal environmental condition we failed to notice any such change in the *Tc_Wap^{F}* knockdown females. Eggs are known to possess their own defence systems against microbial infection (Gantois et al. 2009), we further questioned if *Tc_Wap^{F}* acts as a line of defense for the eggs laid by *Tribolium* female adults. For this the development of eggs laid by *Tc_Wap^{F}* knockdown and control females was tracked and compared. Slightly higher number of eggs were laid by *Tc_Wap^{F}* knockdown females as compared to that of control females. At this moment we don't know the reason for the same, but it is possible that there is a tradeoff between female fecundity and immunity provided by Tc_Wap^{F} protein. Interestingly, the eggs laid by *Tc_Wap^{F}* knockdown females failed to hatch and started desiccating after 60-65hrs (normal hatching time of *Tribolium* eggs). Together, the female specific expression of *Tc_Wap^{F}* (in adults), no expression of *Tc_Wap^{F}* in *Tribolium* eggs and failure of hatching of eggs laid by knockdown females suggests the maternal transfer of Tc_Wap^{F} proteins to shield the embryos from environmental pathogens and is crucial for embryonic development. The schematics shown in figure 6 depicts the mode of action of *Tc_Wap^{F}* in *T. castaneum*. Constitutive expression of other two *waprin* genes (*Tc_Wap^{2}* and *Tc_Wap^{3}*) might be involved in protecting all the *Tribolium* stages from the environmental pathogens.

The central axis (*tra-dsx*) of insect sex determination cascade is conserved in *T. castaneum* also (Shukla and Palli 2012b). Sex-specifically spliced *Tcdsx* produces sex-specific proteins (sex-specific transcription factors- TcDsx^{m} in males and TcDsx^{f} in females) in the two sexes

which is known to regulate all the genes expressed in a sex-specific manner (Shukla and Palli 2012a). To explore whether the sex-specific expression of *Tc_Wap*^{F} gene is regulated by female-specific TcDsx, we examined the presence of TcDsx binding sites in upstream region of *Tc_Wap*^{F} gene. Even though the upstream region (4.5 Kbp upstream of the *Tc_Wap*^{F} transcription start site) of *Tc_Wap*^{F} harbors a conserved Dsx-binding motif (ACAATGT), neither the decreased expression of *Tc_Wap*^{F} was found in *Tcdsx* knockdown females nor there was increased expression of *Tc_Wap*^{F} in *Tcdsx* knockdown males suggesting that TcDsx is not involved in regulating the female specific expression of *Tc_Wap*^{F}.

We further hypothesized that the transcription of *Tc_Wap*^{F} might be regulated by another (yet to discover) transcription factor regulated by TcTra, a splicing regulator of *Tcdsx*. To rule out this possibility, we performed the knockdown of *Tctra* in female adults but still no change in the expression of *Tc_Wap*^{F} was noticed in *Tctra* knockdown females. Hence, we conclude that the sex-specific expression of *Tc_Wap*^{F} is not under the control of the established sex determination pathway of *T. castaneum*. It is possible that the sex-specific expression of *Tc_Wap*^{F} is regulated by a pathway bifurcated at the most upstream gene (yet to be discovered) of *Tribolium* sex determination cascade. Alternatively, we also presume the involvement of chromatin remodelling genes in the female-specific expression of *Tc_Wap*^{F}. Our hypothesis is in line with a recent report by Nanni et al., 2023 which suggests the association of sex-biased expression of genes with differential state (open or closed state) of chromatin in the two sexes of *Drosophila melanogaster* and *D. simulans* (Nanni et al. 2023). Therefore, the sex-specific expression of *Tc_Wap*^{F} gene could be because of open chromatin in female and closed chromatin in males, in *T. castaneum*.

In conclusion, we identified *Tc_Wap*^{F}, a distinct member of WAP family. To our knowledge, this study represents the first report of identification of sex-specific *waprin* from any insect species besides deducing its biological function in the development of *Tribolium*. Interestingly, *Tc_Wap*^{F} genes has the potential to be used in dsRNA mediated pesticide. It will be interesting to study the comparative microbicidal properties of *Tc_Wap* genes in future. The properties like anti-microbial can be exploited further to develop new strategies to counteract the antimicrobial resistance which will add value to the pharmacological applications.

6. Author's Contribution:

CC, VV and JNS conceived the idea of manuscript. CC performed experiments of knockdown, PCR and qRT-PCR. SS assisted CC in experiments and manuscript writing. DK did the phylogenetic analysis and domain search. KKM helped with all the figures and tables in the manuscript besides maintaining the insect cultures. CC and JNS wrote the manuscript and all others agreed to it. All authors contributed to the article and approved the submitted version.

7. Acknowledgements:

J N Shukla acknowledges Ramalingaswami grant (BT/RLF/ Re-entry/10/2015) from Department of Biotechnology, New Delhi and EMR grant (EMR/2017/001378/AS) from DST-SERB, New Delhi for their generous support. Sincere acknowledgement to the Department of Biotechnology and Department of Sport Sciences, Central University of Rajasthan for providing the infrastructural facilities to carry out this work. VV is a Ramalingaswami fellow and, CC and KKM are the Ph.D. students at CURAJ. SS and DK were M.Sc. Biotechnology students of CURAJ. JNS also acknowledges Dr. Satnam Singh (Punjab Agricultural University, Ludhiana) and Dr. Deepa Agashe (National Centre for Biological Sciences, Bangalore) for providing us the *Tribolium castaneum* culture.

8. References:

Alam, Md. Iqbal, Ojha R, Alam MA, Quasimi H, and Alam O. 2019. "Therapeutic Potential of Snake Venoms as Antimicrobial Agents." *Frontiers in Drug, Chemistry and Clinical Research* 2 (4). https://doi.org/10.15761/fdccr.1000136.

Ali, Mohamed F, Karen R Lips, Floyd C Knoop, Bernd Fritzsch, Caroline Miller, and J Michael Conlon. 2002. "Antimicrobial Peptides and Protease Inhibitors in the Skin Secretions of the Crawfish Frog, Rana Areolata." *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* 1601 (1): 55–63.

Bouzid, Wafa, Marion Verdenaud, Christophe Klopp, Frédéric Ducancel, Céline Noirot, and Angélique Vétillard. 2014. "De Novo Sequencing and Transcriptome Analysis for Tetramorium Bicarinatum: A Comprehensive Venom Gland Transcriptome Analysis

from an Ant Species." *BMC Genomics* 15 (1). https://doi.org/10.1186/1471-2164-15-987.

Campbell, James F, Christos G Athanassiou, David W Hagstrum, and Kun Yan Zhu. 2021. "Annual Review of Entomology Tribolium Castaneum: A Model Insect for Fundamental and Applied Research." https://doi.org/10.1146/annurev-ento-080921.

Chen, Dandan, Nanhai He, and Xun Xu. 2008. "Mj-DWD, a Double WAP Domain-Containing Protein with Antiviral Relevance in Marsupenaeus Japonicus." *Fish and Shellfish Immunology* 25 (6): 775–81. https://doi.org/10.1016/j.fsi.2008.02.017.

Coutts, M P. 1969a. "The Mechanism of Pathogenicity of Sirex Noctilio on Pinus Radiata I. Effects of the Symbiotic Fungus Amylostereum Sp. (Thelophoraceae)." *Australian Journal of Biological Sciences* 22 (4): 915–24. https://doi.org/10.1071/BI9690915.

Coutts, M P. 1969b. "The Mechanism of Pathogenicity of Sirex Noctilio on Pinus Radiata II. Effects of S. Noctilio Mucus." *Australian Journal of Biological Sciences* 22 (5): 1153–62. https://doi.org/10.1071/BI9691153.

Gantois, Inne, Richard Ducatelle, Frank Pasmans, Freddy Haesebrouck, Richard Gast, Tom J Humphrey, and Filip Van Immerseel. 2009. "Mechanisms of Egg Contamination by Salmonella Enteritidis." *FEMS Microbiology Reviews* 33 (4): 718–38. https://doi.org/10.1111/j.1574-6976.2008.00161.x.

Gao, Chenglong, Lili Ren, Ming Wang, Zhengtong Wang, Ningning Fu, Huiying Wang, Xiaochen Wang, et al. 2021. "Proteo-Transcriptomic Characterization of Sirex Nitobei (Hymenoptera: Siricidae) Venom." *Toxins* 13 (8). https://doi.org/10.3390/toxins13080562.

Hagiwara, Koichi, Tohru Kikuchi, Yoshiyuki Endo, Huqun, Kazuhiro Usui, Mitsu Takahashi, Naoko Shibata, et al. 2003. "Mouse SWAM1 and SWAM2 Are Antibacterial Proteins Composed of a Single Whey Acidic Protein Motif." *The Journal of Immunology* 170 (4): 1973–79. https://doi.org/10.4049/jimmunol.170.4.1973.

Hauton, C., V. Brockton, and V. J. Smith. 2006. "Cloning of a Crustin-like, Single Whey-Acidic-Domain, Antibacterial Peptide from the Haemocytes of the European Lobster, Homarus Gammarus, and Its Response to Infection with Bacteria." *Molecular Immunology* 43 (9): 1490–96. https://doi.org/10.1016/j.molimm.2005.07.029.

Imjongjirak, Chanprapa, Piti Amparyup, Anchalee Tassanakajon, and Siriporn Sittipraneed. 2009. "Molecular Cloning and Characterization of Crustin from Mud Crab Scylla Paramamosain." *Molecular Biology Reports* 36 (5): 841–50. https://doi.org/10.1007/s11033-008-9253-0.

Klompen, Anna M.L., Jason Macrander, Adam M. Reitzel, and Sérgio N. Stampar. 2020. "Transcriptomic Analysis of Four Cerianthid (Cnidaria, Ceriantharia) Venoms." *Marine Drugs* 18 (8). https://doi.org/10.3390/MD18080413.

Lee, Kwang Sik, Bo Yeon Kim, Yun Hui Kim, Yong Soo Choi, and Byung Rae Jin. 2023. "Identification of Waprin and Its Microbicidal Activity: A Novel Protein Component of Honeybee (Apis Mellifera) Venom." *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology* 266 (April). https://doi.org/10.1016/j.cbpc.2023.109561.

Li, Shuang, Xing Kun Jin, Xiao Nv Guo, Ai Qing Yu, Min Hao Wu, Shang Jian Tan, You Ting Zhu, Wei Wei Li, and Qun Wang. 2013. "A Double WAP Domain-Containing Protein Es-DWD1 from Eriocheir Sinensis Exhibits Antimicrobial and Proteinase Inhibitory Activities." *PLoS ONE* 8 (8). https://doi.org/10.1371/journal.pone.0073563.

Liu, Daixi, Yuwei Wang, Lin Wei, Huahu Ye, Huan Liu, Ling Wang, Rui Liu, Dongsheng Li, and Ren Lai. 2013. "Snake Venom-like Waprin from the Frog of Ceratophrys Calcarata Contains Antimicrobial Function." *Gene* 514 (2): 99–104. https://doi.org/10.1016/j.gene.2012.11.007.

Livak, Kenneth J., and Thomas D. Schmittgen. 2001. "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method." *Methods* 25 (4): 402–8. https://doi.org/10.1006/meth.2001.1262.

Nair, Dileep G., Bryan G. Fry, Paul Alewood, Prakash P. Kumar, and R. Manjunatha Kini. 2007. "Antimicrobial Activity of Omwaprin, a New Member of the Waprin Family of Snake Venom Proteins." *Biochemical Journal* 402 (1): 93–104. https://doi.org/10.1042/BJ20060318.

Nanni, Adalena V, Natalie Martinez, Rita Graze, Alison Morse, Jeremy R B Newman, Vaibhav Jain, Srna Vlaho, et al. 2023. "Sex-Biased Expression Is Associated With Chromatin State in Drosophila Melanogaster and Drosophila Simulans." *Molecular Biology and Evolution* 40 (5): msad078. https://doi.org/10.1093/molbev/msad078.

Pierre, L. St., S. T. Earl, I. Filippovich, N. Sorokina, P. P. Masci, J. De Jersey, and M. F. Lavin. 2008. "Common Evolution of Waprin and Kunitz-like Toxin Families in Australian Venomous Snakes." *Cellular and Molecular Life Sciences* 65 (24): 4039–54. https://doi.org/10.1007/s00018-008-8573-5.

Reeks, T. A., B. G. Fry, and P. F. Alewood. 2015. "Privileged Frameworks from Snake Venom." *Cellular and Molecular Life Sciences* 72 (10): 1939–58. https://doi.org/10.1007/s00018-015-1844-z.

Romero-Gutiérrez, Maria Teresa, Carlos Eduardo Santibáñez-López, Juana María Jiménez-Vargas, Cesar Vicente Ferreira Batista, Ernesto Ortiz, and Lourival Domingos Possani. 2018. "Transcriptomic and Proteomic Analyses Reveal the Diversity of Venom Components from the Vaejovid Scorpion Serradigitus Gertschi." *Toxins* 10 (9). https://doi.org/10.3390/toxins10090359.

Romero-Gutierrez, Teresa, Esteban Peguero-Sanchez, Miguel A. Cevallos, Cesar V.F. Batista, Ernesto Ortiz, and Lourival D. Possani. 2017. "A Deeper Examination of Thorellius Atrox Scorpion Venom Components with Omic Techonologies." *Toxins* 9 (12). https://doi.org/10.3390/toxins9120399.

JN Shukla, and Subba Reddy Palli. 2012a. "Doublesex Target Genes in the Red Flour Beetle, Tribolium Castaneum." *Scientific Reports* 2. https://doi.org/10.1038/srep00948.

JN Shukla, and Subba Reddy Palli. 2012b. "Sex Determination in Beetles: Production of All Male Progeny by Parental RNAi Knockdown of Transformer." *Scientific Reports* 2. https://doi.org/10.1038/srep00602.

JN Shukla, and Subba Reddy Palli. 2013. "Tribolium Castaneum Transformer-2 Regulates Sex Determination and Development in Both Males and Females." *Insect Biochemistry and Molecular Biology* 43 (12): 1125–32. https://doi.org/10.1016/j.ibmb.2013.08.010.

JN Shukla, and Subba Reddy Palli. 2014. "Production of All Female Progeny: Evidence for the Presence of the Male Sex Determination Factor on the y Chromosome." *Journal of Experimental Biology* 217 (10): 1653–55. https://doi.org/10.1242/jeb.100438.

Smith, Valerie J., Jorge M.O. Fernandes, Graham D. Kemp, and Chris Hauton. 2008. "Crustins: Enigmatic WAP Domain-Containing Antibacterial Proteins from

Crustaceans." *Developmental and Comparative Immunology*. https://doi.org/10.1016/j.dci.2007.12.002.

Suntravat, Montamas, Néstor L. Uzcategui, Chairat Atphaisit, Thomas J. Helmke, Sara E. Lucena, Elda E. Sánchez, and Alexis Rodríguez Acosta. 2016. "Gene Expression Profiling of the Venomgland from the Venezuelan Mapanare (Bothrops Colombiensis) Using Expressed Sequence Tags (ESTs)." *BMC Molecular Biology* 17 (1). https://doi.org/10.1186/s12867-016-0059-7.

Thankappan, Bency, and Jayaraman Angayarkanni. 2019. "Biological Characterization of Omw1 and Omw2: Antimicrobial Peptides Derived from Omwaprin." *3 Biotech* 9 (8). https://doi.org/10.1007/s13205-019-1801-x.

Torres, Allan M., Hui Y. Wong, Malan Desai, Shabbir Moochhala, Philip W. Kuchel, and R. Manjunatha Kini. 2003. "Identification of a Novel Family of Proteins in Snake Venoms. Purification and Structural Characterization of Nawaprin from Naja Nigricollis Snake Venom." *Journal of Biological Chemistry* 278 (41): 40097–104. https://doi.org/10.1074/jbc.M305322200.

Toutges, Michelle J., Kris Hartzer, Jeffrey Lord, and Brenda Oppert. 2010. "Evaluation of Reference Genes for Quantitative Polymerase Chain Reaction across Life Cycle Stages and Tissue Types of Tribolium Castaneum." *Journal of Agricultural and Food Chemistry* 58 (16): 8948–51. https://doi.org/10.1021/jf101603j.

Zhang, Xiaoming, Raymond P. Boot-Handford, Julie Huxley-Jones, Lorna N. Forse, A. Paul Mould, David L. Robertson, Li Li, Matthews Athiyal, and Michael P. Sarras. 2007. "The Collagens of Hydra Provide Insight into the Evolution of Metazoan Extracellular Matrices." *Journal of Biological Chemistry* 282 (9): 6792–6802. https://doi.org/10.1074/jbc.M607528200.

Figure Legends:

Figure 1: Temporal and spatial expression profiles of *waprin* genes (*Tc_Wap*^{F} ,*Tc_Wap*^{2} and *Tc_Wap*^{3}) of *T. castaneum*.

A) Developmental profiling of *waprin* genes of *T. castaneum*. Semiquantitative RT-PCR was performed using stage specific cDNAs (6th Instar Larvae, Q Stage, Larvae, female pupae, male pupae, female adult and male adults) as templates. *Tc_Wap*^{F} shows female exclusive expression while the other two *waprin* genes (*Tc_Wap*^{2} and *Tc_Wap*^{3}) express at all the stages. B) Tissue

specific expression profile of *Tc_Wap^F*; *Tc_Wap^F* expresses in all the tissues of adult female. C) Expression profile of *waprin* genes in developing embryos (from 2 hours to 60 hours after egg laying). RPS-18 gene was used as an endogenous control. D) Relative expression of *Tc_Wap^F* in 6th day old adult male and female. qRT-PCR analysis was performed using the sex specific cDNA's as templates. Error bars represent the standard deviation (p value=0.0035).

Figure 2A: qRT-PCR analysis to check the expression of *Tc_Wap^F* in the knockdown females and control females. The templates used were cDNA of control (*ds_GFP* injected) and knockdown (*Tc_Wap^F* injected) females. Error bars represent s.d. **p value≤ 0.0035.

Figure 2B: Graphical representation of number of individuals injected and number of individuals survived after dsRNA injection versus the number of eggs laid by the survived females. A slight increase in egg laying was observed by *Tc_Wap^F* knockdown females as comparison to control females. (C1-C4: Represents control (ds_GFP injected) females; W1-W4: Represents knockdown (*Tc_Wap^F*) females.)

Figure 2C: Picture of eggs laid by control and *Tc_Wap^F* knockdown females.

Figure 3A: Graphs depicting the knockdown efficiency of *Tcdsx*. dsRNA injections targeting common region of *Tcdsx* were performed separately in female and male adults (0 day). *p value≤ 0.0311; **p value ≤ 0.0003

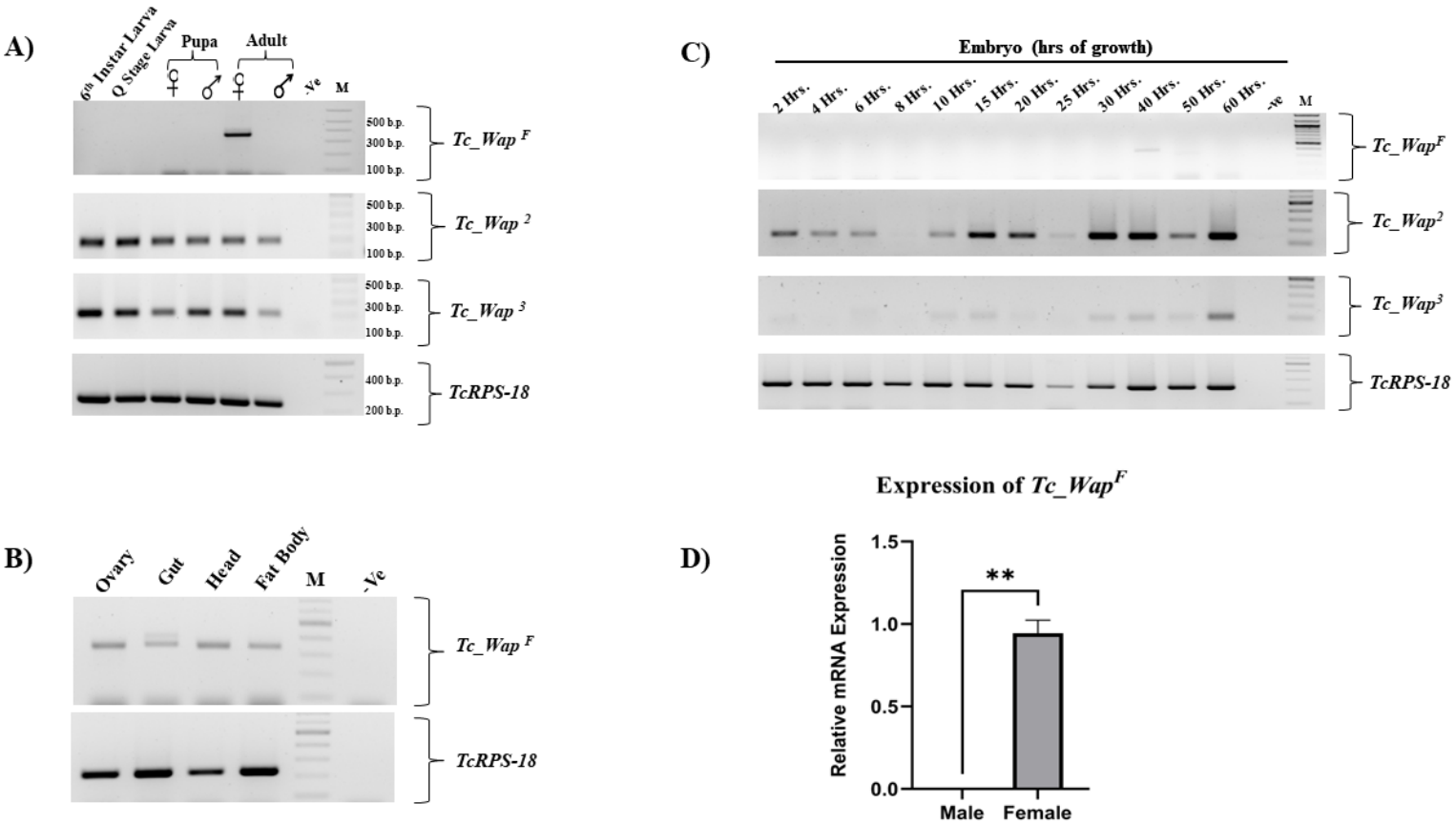
Figure 3B: Relative expression of *Tc_Wap^F* in response to *Tcdsx* knockdown, in female and male individuals. Five biological replicates were used in each experiment. Error bars represent standard deviation.

Figure 3C: Graphical representation of the effect of *Tc_Tra* knockdown on the expression of *Tc_Wap^F*. The knockdown efficiency of *Tc_Tra* and the expression of *Tc_Wap^F* was analysed both at 3rd day and 5th day after ds*Tctra* injections.

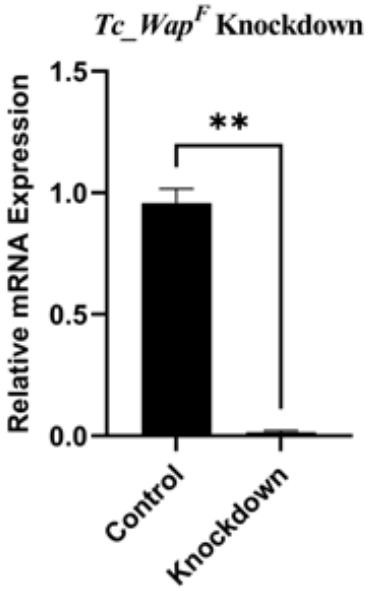
Figure 4: Phylogenetic analysis of WAP proteins across seven different orders of class 'insecta'. The circular phylogenetic tree was constructed with the MEGA11 program using the neighbour-joining method.

Figure 5: Domain architecture of various WAP domain containing proteins across different insect species. Different domains are colour coded and mentioned in the right side of the picture.

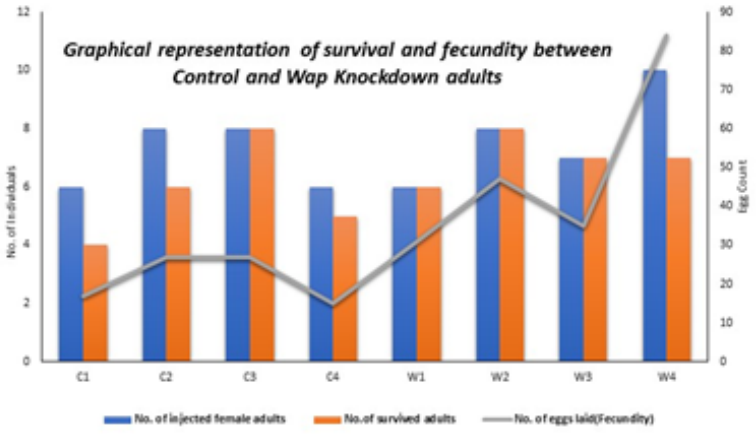
Figure 6: Schematics showing the mode of action of *Tc_Wap^F*. *Tc_Wap^F* is maternally transferred to next generation. But when *Tc_Wap^F* is silenced through dsRNA mediated knockdown, the maternal transcripts get degraded and therefore the embryos failed to hatch.


A)
6th Instar Larva
Q Stage Larva
Pupa
Adult
♀
♂
♀
♂
-Ve
M
500 b.p.
300 b.p.
100 b.p.
Tc_Wap F
Tc_Wap 2
Tc_Wap 3
400 b.p.
200 b.p.
TcRPS-18
B)
Ovary
Gut
Head
Fat Body
M
-Ve
Tc_Wap F
TcRPS-18
C)
Embryo (hrs of growth)
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
10 Hrs.
15 Hrs.
20 Hrs.
25 Hrs.
30 Hrs.
40 Hrs.
50 Hrs.
60 Hrs.
-ve
M
Tc_WapF
Tc_Wap2
Tc_Wap3
TcRPS-18
D)
Expression of Tc_WapF
Relative mRNA Expression
1.5
1.0
0.5
0.0
**
Male
Female

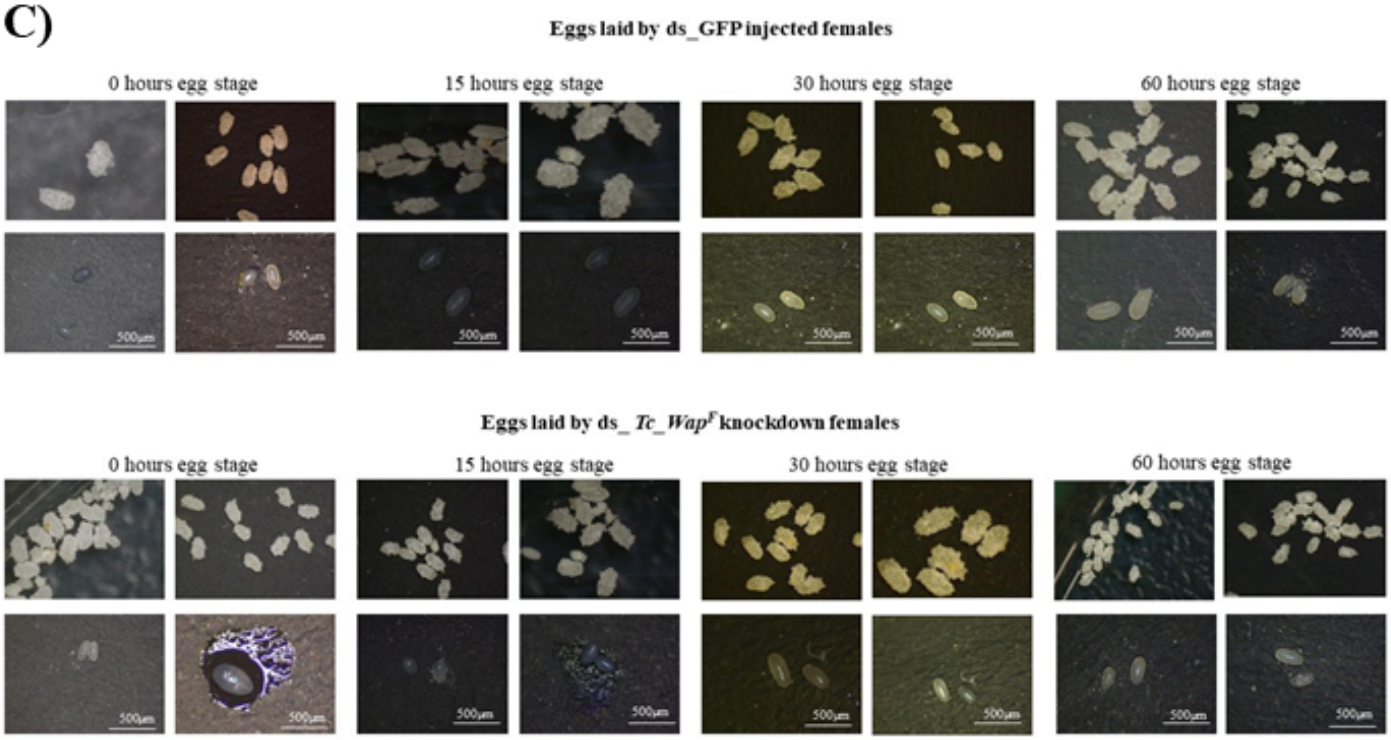
A)


Tc_Wap^F Knockdown
Relative mRNA Expression
1.5
1.0
0.5
0.0
**
Control
Knockdown

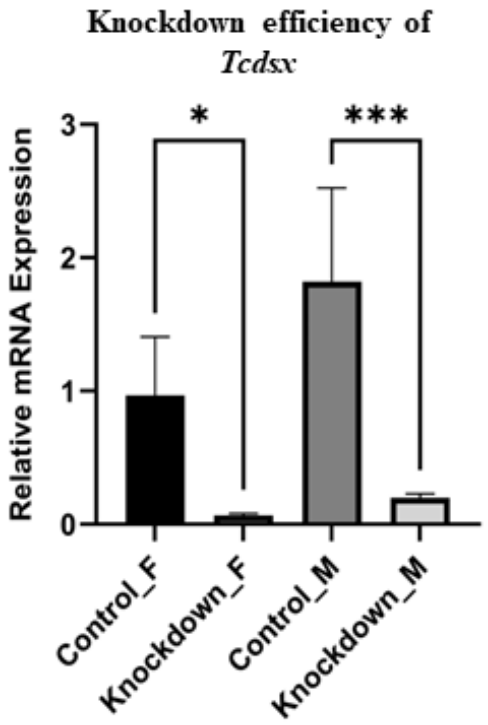
B)


Graphical representation of survival and fecundity between Control and Wap Knockdown adults
No. of Individuals
Egg Count
C1
C2
C3
C4
W1
W2
W3
W4
No. of injected female adults
No.of survived adults
No. of eggs laid(Fecundity)

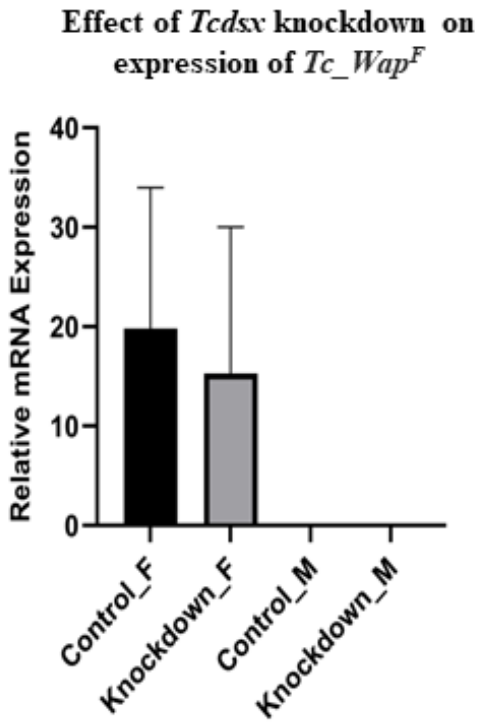
C)


Eggs laid by ds_GFP injected females
0 hours egg stage
15 hours egg stage
30 hours egg stage
60 hours egg stage
500µm
Eggs laid by ds_ Tc_Wap^F knockdown females
0 hours egg stage
15 hours egg stage
30 hours egg stage
60 hours egg stage
500µm

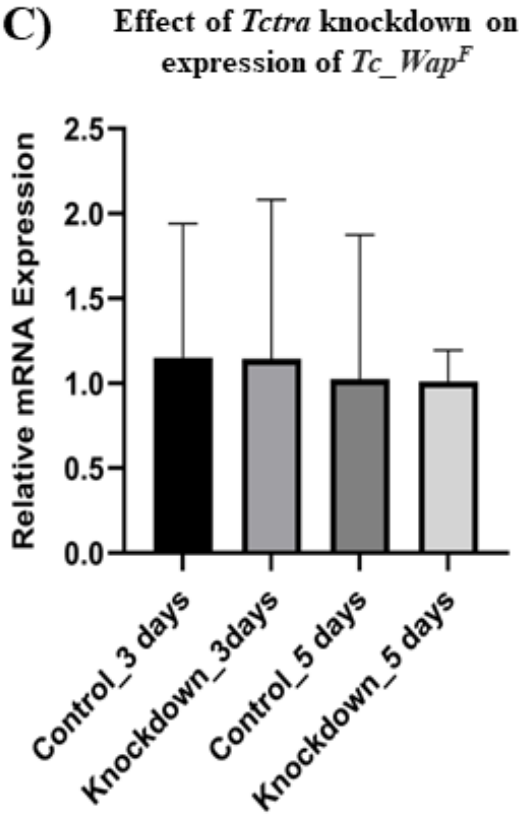
A)

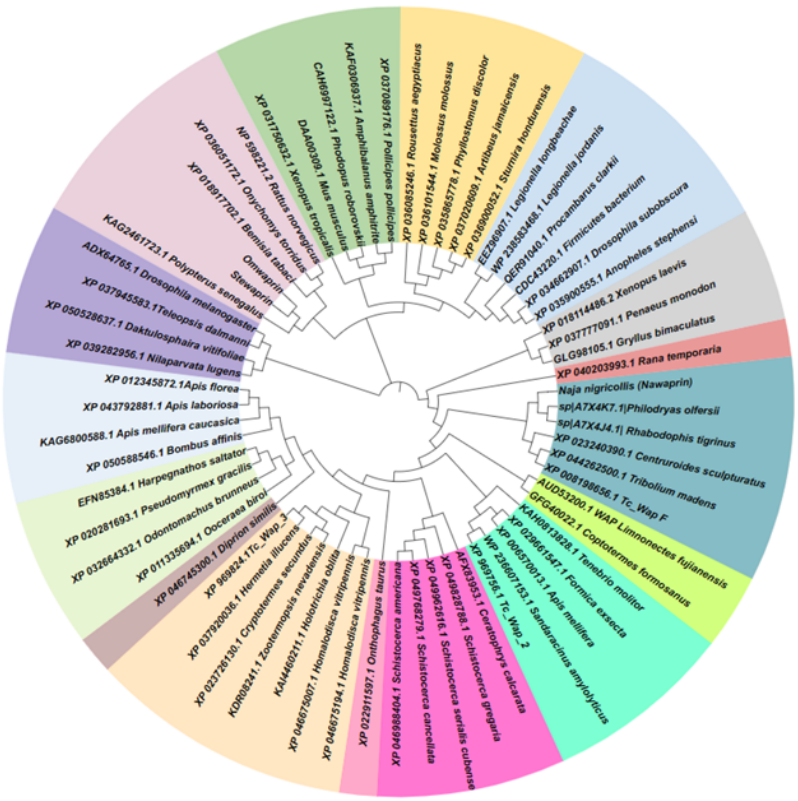

Knockdown efficiency of
Tcdsx
Relative mRNA Expression
3
2
1
0
*
***
Control_F
Knockdown_F
Control_M
Knockdown_M

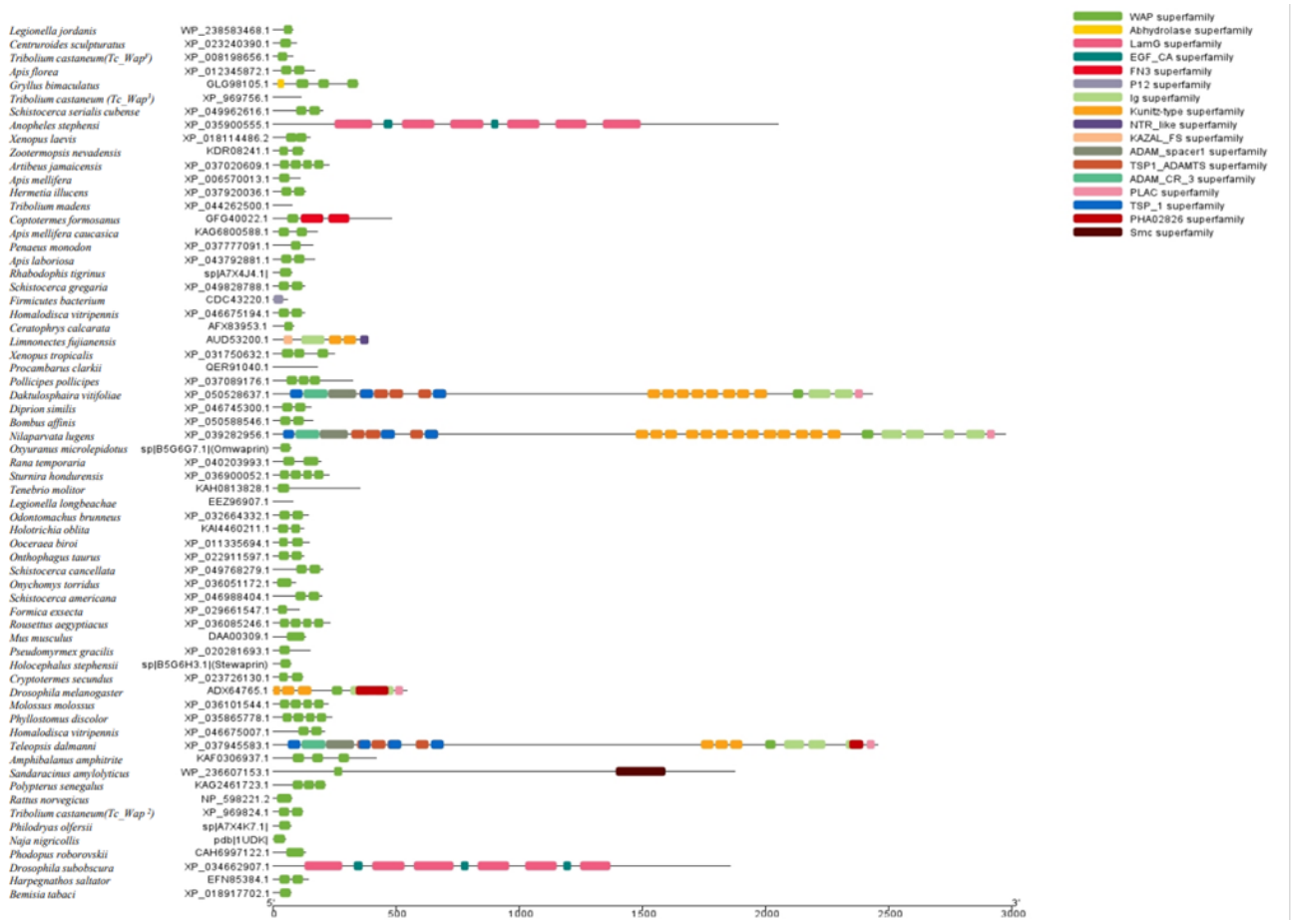
B)

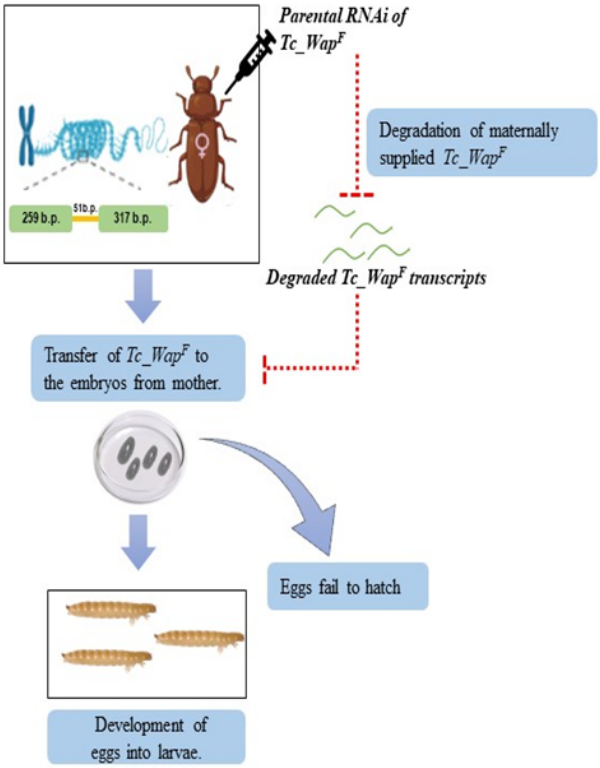

Effect of Tcdsx knockdown on
expression of Tc_WapF
Relative mRNA Expression
40
30
20
10
0
Control_F
Knockdown_F
Control_M
Knockdown_M

C)


Effect of Tctra knockdown on
expression of Tc_WapF
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
Control_3 days
Knockdown_3days
Control_5 days
Knockdown_5 days


XP 037089776.1 Podilicipes podiceps
KAF0306937.1 Amphitalanus amphitrite
CAN6971122.1 Phodopus roborovskii
DAA00309.1 Mus musculus
NP 598221.2 Rattus norvegicus
XP 031756632.1 Xenopus tropicalis
XP 036051172.1 Onychomys torridus
XP 018917702.1 Burmisa tabaci
Onwaprin
Stewaprin
KAG2461723.1 Polypterus senegalus
ADX64765.1 Drosophila melanogaster
XP 037945583.1Teleopsis dalmanni
XP 050528637.1 Daktulosphaira vitifoliae
XP 039282956.1 Nilaparvata lugens
XP 012345872.1Apis florea
XP 043792881.1 Apis laboriosa
KAG6800588.1 Apis mellifera caucasica
XP 050588546.1 Bombus affinis
EFN85384.1 Harpegnathos saltator
XP 020281693.1 Pseudomyrmex gracilis
XP 032664332.1 Odontomachus brunneus
XP 011335694.1 Ooceraea biroi
XP 046745300.1 Diprion similis
XP 969824.1Tc_Wap_3
XP 037920036.1 Hermetia illucens
XP 037326130.1 Cryptotermes secundus
KDR08241.1 Zootermopsis nevadensis
KAI4460211.1 Holotrichia oblita
XP 046675007.1 Homalodisca vitripennis
XP 045675194.1 Homalodisca vitripennis
XP 022911597.1 Onthophagus taurus
XP 046988404.1 Schistocerca americana
XP 049763273.1 Schistocerca cancellata
XP 049962616.1 Schistocerca gregaria
XP 049828788.1 Schistocerca serialis cubense
XP 039393.1 Cerehophys calcarata
WP 298758.1 Tc_Wap_2
XP 036071133.1 Sundaracinus amylolyticus
XP 005570013.1 Apis mellifera
XP 039601547.1 Formica exsecta
KAH0813828.1 Tenebrio molitor
GFG40022.1 Coptotermes formosanus
AUD53200.1 WAP Limnonectes fujianensis
XP 008198656.1 Tc_Wap F
XP 044262500.1 Centruroides sculpturatus
XP 023240390.1 Rhabdophis tigrinus
sp|A7X4J4.1| Rhabdophis tigrinus
sp|A7X4K7.1|Philodryas olfersii
Naja nigricollis (Nawaprin)
XP 040203993.1 Rana temporaria
GLG98105.1 Gryllus bimaculatus
XP 037777091.1 Penaeus monodon
XP 018114486.2 Xenopus laevis
XP 035900555.1 Anopheles stephensi
XP 034662907.1 Drosophila subobscura
CDC43220.1 Procambarus clarkii
OER91040.1 Legionella jordanis
WP 238583468.1 Legionella longbeachae
EEZ56907.1 Stomoxys hondurensis
XP 037020609.1 Artibeus jamaicensis
XP 035965778.1 Phyllostomus discolor
XP 036101544.1 Molossus molossus
XP 036085246.1 Rousettus aegyptiacus


Legionella jordanis WP_238583468.1
Centruroides sculpturatus XP_023240390.1
Tribolium castaneum(Tc_Wap²) XP_008198656.1
Apis florea XP_012345872.1
Gryllus bimaculatus GLG98105.1
Tribolium castaneum (Tc_Wap¹) XP_969756.1
Schistocerca serialis cubense XP_049962616.1
Anopheles stephensi XP_035900555.1
Xenopus laevis XP_018114486.2
Zootermopsis nevadensis KDR08241.1
Artibeus jamaicensis XP_037020609.1
Apis mellifera XP_006570013.1
Hermetia illucens XP_037920036.1
Tribolium madens XP_044262500.1
Coptotermes formosanus GFG40022.1
Apis mellifera caucasica KAG6800588.1
Penaeus monodon XP_037777091.1
Apis laboriosa XP_043792881.1
Rhabodophis tigrinus sp|A7X4J4.1|
Schistocerca gregaria XP_049828788.1
Firmicutes bacterium CDC43220.1
Homalodisca vitripennis XP_046675194.1
Ceratophrys calcarata AFX83953.1
Limnonectes fujianensis AUD53200.1
Xenopus tropicalis XP_031750632.1
Procambarus clarkii QER91040.1
Pollicipes pollicipes XP_037089176.1
Daktulosphaira vitifoliae XP_050528637.1
Diprion similis XP_046745300.1
Bombus affinis XP_050588546.1
Nilaparvata lugens XP_039282956.1
Oxyuranus microlepidotus sp|B5G6G7.1|(Omwaprin)
Rana temporaria XP_040203993.1
Sturnira hondurensis XP_036900052.1
Tenebrio molitor KAH0813828.1
Legionella longbeachae EEZ96907.1
Odontomachus brunneus XP_032664332.1
Holotrichia oblita KAI4460211.1
Ooceraea biroi XP_011335694.1
Onthophagus taurus XP_022911597.1
Schistocerca cancellata XP_049768279.1
Onychomys torridus XP_036051172.1
Schistocerca americana XP_046988404.1
Formica exsecta XP_029661547.1
Rousettus aegyptiacus XP_036085246.1
Mus musculus DAA00309.1
Pseudomyrmex gracilis XP_020281693.1
Holocephalus stephensii sp|B5G6H3.1|(Stewaprin)
Cryptotermes secundus XP_023726130.1
Drosophila melanogaster ADX64765.1
Molossus molossus XP_036101544.1
Phyllostomus discolor XP_035865778.1
Homalodisca vitripennis XP_046675007.1
Teleopsis dalmanni XP_037945583.1
Amphibalanus amphitrite KAF0306937.1
Sandaracinus amylolyticus WP_236607153.1
Polypterus senegalus KAG2461723.1
Rattus norvegicus NP_598221.2
Tribolium castaneum(Tc_Wap²) XP_969824.1
Philodryas olfersii sp|A7X4K7.1|
Naja nigricollis pdb|1UDK|
Phodopus roborovskii CAH6997122.1
Drosophila subobscura XP_034662907.1
Harpegnathos saltator EFN85384.1
Bemisia tabaci XP_018917702.1
5'
0
500
1000
1500
2000
2500
3000
3'
WAP superfamily
Abhydrolase superfamily
LamG superfamily
EGF_CA superfamily
FN3 superfamily
P12 superfamily
Ig superfamily
Kunitz-type superfamily
NTR_like superfamily
KAZAL_FS superfamily
ADAM_spacer1 superfamily
TSP1_ADAMTS superfamily
ADAM_CR_3 superfamily
PLAC superfamily
TSP_1 superfamily
PHA02826 superfamily
Smc superfamily


Parental RNAi of
Tc_Wap^F
259 b.p.
51b.p.
317 b.p.
Degradation of maternally supplied Tc_Wap^F
Degraded Tc_Wap^F transcripts
Transfer of Tc_Wap^F to the embryos from mother.
Eggs fail to hatch
Development of eggs into larvae.