A Sex specific homologue of snake Waprin is essential for Embryonic Development in the Red Flour Beetle, *Tribolium castaneum*

Running Title: Egg development in *Tribolium* requires Waprin

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Summary statement:

Tribolium castaneum possesses a female-specific homologue of snake Waprin (Tc_Wap^{F}) which is essential for its embryonic development. Expression of *Tc_Wap^{F}* is independent of *Tribolium* sex-determination cascade.

1. Abstract:

Waprin, an extracellular secretory protein, is widely known for its antibacterial activity. Through the analysis of sex-specific transcriptomes of adult *Tribolium castaneum*, we identified a homologue of snake *waprin* (*Tc_Wap*^{F}) with significantly higher level of its transcripts in females. Developmental- and tissue-specific profiling revealed the widespread expression of *Tc_Wap*^{F} in adult female tissues. Interestingly, *Tc_Wap*^{F} expression is not regulated by the classical sex-determination cascade of *T. castaneum*, as we fail to get any attenuation in *Tc_Wap*^{F} transcript levels in *Tcdsx* and *Tctra* (key players of sex determination cascade of *T. castaneum*) knockdown females. The eggs laid by *Tc_Wap*^{F} knockdown females never hatched, indicating the involvement of *Tc_Wap*^{F} in the embryonic development in *T. castaneum*. This is the first report of identification a sex-specific *waprin* homologue in an insect and its involvement as a novel candidate in the embryonic development. Future investigations on the functional conservation of insect *waprins* and their mechanistic role in embryonic development can be exploited for improving pest management strategies.

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). Subsequent studies demonstrated the ubiquitous nature of Waprin proteins across genera (Alam et al., 2019). These proteins are mostly found in the extracellular secretions of mammals such as whey protein in milk (Piletz et al., 1981), as well as in snakes (St. Pierre et al., 2008; Reeks et al., 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) among others. Additionally, *waprin*-like genes have also been reported in the transcriptomes of several other organisms, like cnidarians (Klompen et al., 2020) and ants (Bouzid et al., 2014). A thorough structural and functional characterization of the 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 done. This led to the identification of eight cysteine residues at the core of Waprin protein, which facilitate the formation of four disulphide bonds (Suntravat et al., 2016; Alam et al., 2019).

The WAP-domain containing proteins are involved in multifarious activities including antiviral activity (Chen et al., 2008), antimicrobial property (Ali et al., 2002; Hagiwara et al., 2003; Hauton et al., 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),and immunomodulatory functions (Gao et al., 2021). The most studied function of Waprin proteins include its antimicrobial activity against various gram-positive and gram-negative bacteria and its antifungal activity against certain fungi (Liu et al., 2013). Ceratoxin isolated 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) from *O. microlepidotus* specifically targets gram-negative bacterial strains like *Staphylococcus warneri* and *Bacillus megaterium* (Nair et al., 2007). The antimicrobial activity of Omwaprin remains stable under high salt concentration (up to 350mM NaCl) and is dependent upon its eight cysteine residues or its six N-terminal residues, four of which are positively charged. This suggested the critical role of the four disulphide bonds within the core of Waprin structure for its antibacterial activity (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 encoding Waprin-like proteins is, so far, limited only to a few insect species, such as, guinea ant, *Tetramorium bicarinatum* (Bouzid et al., 2014), wood-boring woodwasp, *Sirex nitobei* (Gao et al., 2021), and honeybee, *Apis mellifera* (Lee et al., 2023). While the function of *waprin* in *T. bicarinatum* remains elusive, *waprin* of *S. nitobei* is hypothesized to promote the growth of a symbiotic fungus, *Amylosterium areolatum*, by exercising antibacterial activities. During oviposition, female *S. nitobei* 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 while *waprin* in the venom inhibits the bacterial growth, facilitating the growth of *A. areolatum* (Coutts, 1969a, 1969b; Gao et al., 2021). However, the recent report of Waprin (Amwaprin) in *A. mellifera* is the first direct evidence about the antibacterial potential of an insect Waprin. Recombinant Amwaprin produced in baculovirus-infected Sf9 cells exhibited microbicidal and anti-elastolytic activities (Lee et al., 2023).

The red flour beetle, *Tribolium castaneum*, is a pest of stored food grains. It's wide geographical range and infestation potential in a variety of stored grains makes it an economically important pest. Additionally, the extent of RNAi efficiency in *T. castaneum* makes it an excellent model for reverse genetics studies (Campbell et al., 2021). Recently,

while analysing sex-specific transcriptomes (unpublished data) of six-day old adult *T. castaneum*, our group identified a female-biased (FPKM value-179.20 and 0.35 for females and males, respectively) transcript of *waprin* (*Tc_Wap*^{F}) gene. *Tc_Wap*^{F} attracted our attention as no sex-specific function of *waprin* has been reported in any organism till date. Therefore, in this study, we characterized *Tc_Wap*^{F} to unravel the significance of its female-exclusive expression. Developmental- and tissue-specific profiling of *Tc_Wap*^{F} confirmed its exclusive expression in adult female tissues. Furthermore, our study revealed that the expression of *Tc_Wap*^{F} is independent of the classical sex determination pathway of *T. castaneum*. Although, we fail to observe any phenotypic alteration in *Tc_Wap*^{F} knockdown females, eggs laid by these females failed to hatch, indicating a direct involvement of *Tc_Wap*^{F} in embryonic development. This pioneering study, reveals a novel aspect of sex-specifically regulated Waprin protein to contribute towards the embryonic development. Further insights into the mechanistic roles of Waprin proteins in the insect kingdom holds promise for their utilization in pest management strategies.

3. Materials and methods

3.1 Rearing of *Tribolium castaneum*

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

3.2 RNA Isolation and RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) from different developmental stages of *Tribolium castaneum* including eggs, 6th instar larvae, Q stage, sex-specific pupae and sex-specific adults. Additionally, total RNA was also isolated from gut, ovary, head and fatbody tissues of female adults. Isolated RNA samples were DNAse (Promega,) treated, and the concentrations of DNA-free RNA samples were determined using NanoDrop (Jenway Genova Nano, USA). Subsequently the RNA samples were stored at -20°C until further use. For cDNA synthesis, 3µg of RNA per sample was denatured at 70°C for 5 min and then immediately chilled on ice. First strand cDNA was synthesized using GoScript Reverse Transcriptase (Promega) with 18-mer oligo(dT) primer, according to the manufacturer's instructions. The prepared cDNA samples were diluted ten folds with nuclease-free water and used for subsequent PCR studies. GoTaq polymerase (Promega) was used for all the RT-PCR reactions

136 using gene-specific primers (Table S1) designed using Primer-3 software (Primer3 Input,
137 version 0.4.0). PCR conditions comprised initial denaturation at 94°C for 2 min followed by
138 32 cycles of denaturation at 94°C for 30 s, annealing (temperatures in Table S1) for 30 s,
139 extension at 72°C for 2 min and a final extension at 72°C for 10 min. The amplified products
140 were electrophoresed on 1.5% agarose gels and visualised using a BioRad gel documentation
141 system.
142
143 **3.3 Sequence analysis**
144 Following the identification of *Tc_Wap*^{F} gene from our transcriptome data (unpublished), its
145 sequence was validated through BLAST (BLASTn) searches in the EST database of *T.*
146 *castaneum*. Genomic DNA corresponding to *waprin* transcript was identified by performing
147 BLAST (BLASTn) searches in the genomic database of *T. castaneum*. Subsequently, the exons
148 and introns of *Tc_Wap*^{F} were identified by aligning the gene-specific EST sequences with their
149 corresponding genomic DNA sequence using Splign software
150 (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi).
151
152 **3.4 Double stranded RNA (dsRNA) synthesis and injections**
153 PCR was performed using cDNA from adult females as template and gene-specific primers
154 (Table S1) containing the T7 promoter sequences at their 5' ends to amplify the specific regions
155 of *Tc_Wap*^{F} (Fig. S1), *Tcdsx* and *Tctra* genes (Shukla and Palli, 2012a, 2012b). Purified
156 amplicons were used as template in in-vitro transcription reaction using T7 RiboMAX™
157 Express RNAi System (Promega) according to the manufacturer's instructions. The control
158 dsRNA was synthesized using an amplified fragment of the *GFP* gene sequence.

159 Newly emerged (0 day) female adults (5 h post adult emergence) were immobilized by keeping
160 them at 4°C for 8–10 min prior to injections. Subsequently, anesthetized insects were then
161 injected either with ds*GFP* (control) or with dsRNA targeting specific genes (*Tc_Wap*^{F}, *Tcdsx*
162 or *Tctra*) using an aspirator tube assembly (Sigma) attached to a 3.50 mm glass capillary tube
163 (Drummond Scientific) drawn by a micropipette puller (Narishige, PC-100). Approximately,
164 500–600 ng dsRNA was injected per insect. Injected insects were starved and allowed to
165 recover at room temperature for around 5 to 6 hours before transferring them to the incubator
166 under standard rearing conditions. Knockdown efficacy of *Tc_Wap*^{F}, *Tcdsx* and *Tctra* were
167 analyzed after 5 days of injections. The ratio of gene expression between beetles injected with

168 target dsRNA and those injected with *GFP* dsRNA was used to calculate the efficacy of gene
169 knockdown in the RNAi insects.
170
171 **3.5 Parental RNAi of *Tc_Wap^F***
172 To investigate the impact of *Tc_Wap^F* knockdown in the subsequent generation, target gene
173 specific dsRNA or control dsRNAs were injected into virgin adult females (0 day) following
174 the protocol described in the previous section (3.4). Subsequently, on 5th day, injected females
175 were allowed to mate with their counterparts (uninjected males of the same age) maintaining
176 one mating pair per cup. After one day of mating, each mating pair was transferred to fresh cup
177 containing fine flour and eggs laid were harvested after every 2 hours to obtain embryos of
178 different developmental stages. The developmental progression of staged eggs was tracked by
179 staining and visualising the dechorionated eggs and, the females (injected with either
180 ds*Tc_Wap^F* or ds*GFP*) were dissected to compare their tissue morphology.
181
182 **3.6 Embryo collection, egg staining and imaging**
183 A series of treatments were given to the staged eggs for embryo staining: initially staged eggs
184 were cleaned by rinsing them in water. Cleaned eggs were treated with 25% bleach solution for
185 1.5 minutes with continuous shaking for their dichlorination. Subsequently, eggs were washed
186 with double distilled water and then fixed in 4% paraformaldehyde solution for 15-20 minutes
187 followed by the removal of aqueous phase. Further, the eggs were vigorously shaken in
188 methanol to remove the vitelline membrane. The devitellinized eggs were then washed with
189 PBST buffer (PBS buffer containing 0.1% Triton X-100). At last, the eggs were stained with
190 Hoechst 33258 (Thermo Fisher) dye and imaged using Leica DMI6000 fluorescence
191 microscope.
192
193 **3.7 qRT-PCR Analysis**
194 Total RNA isolation and first strand cDNA synthesis from control and knockdown females was
195 done as described previously. qRT-PCR reactions were performed using synthesized cDNAs
196 as template and gene-specific primers (Table S1) to quantify the relative mRNA levels of
197 *Tc_Wap^F* with GoTaq® qPCR Master Mix (Promega) according to the manufacturer's
198 instructions.Three independent biological replicates were used for each quantitation. *T.*
199 *castaneum ribosomal protein* (*RPS-18*) gene was used as an endogenous control to normalize
200 the expression data (Toutges et al., 2010), and $2^{-\Delta\Delta CT}$ method was employed to analyze the gene
201 expression levels (Livak and Schmittgen, 2001). The qPCR reactions were carried out in a

202 CFX96 touch real-time PCR detection system (Bio-Rad) with following cycling parameters:
203 initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 45 s
204 and 72 °C for 5s. Melt curve analysis was carried out in the temperature range of 60–95°C, in
205 steps of 0.5 °C increments per 5s.
206
207 **3.8 Phylogenetic Analysis and Domain Architecture**
208 Protein sequence of *T. castaneum* Waprin (XP_008198656.1) was used to perform BLAST
209 searches (BLAST-p) in the non-redundant (nr) protein database of NCBI. The protein
210 sequences of different insects and few other organisms (including bacteria, crustaceans,
211 scorpions, reptiles, amphibians and mammals) were aligned by MUSCLE and the aligned
212 sequences were used to construct the phylogenetic tree using the Maximum likelihood method
213 in MEGA 11.0. Phylogenetic analysis was performed using the iTOL tool which recognizes the
214 Newick file. Domain searches and motif scan were conducted using online softwares,
215 Conserved domain database (https://www.ncbi.nlm.nih.gov/cdd/) and motif scan
216 (https://myhits.sib.swiss/cgi-bin/motif_scan), respectively. Eight conserved cysteine residues
217 were used as a signature to identify the conserved domains and establish the relationship
218 between their associations and divergence.
219
220 **3.9 Statistical Analysis**
221 The statistical analysis was performed using GraphPad Prism (9.5.1). *t-test* was performed to
222 analyse the *Tc_Wap*^{F} gene expression and its knockdown efficiency while one way ANOVA
223 were used to compare the *TcTra* and *Tcdsx* gene expression between the control and knockdown
224 samples. 3-4 biological replicates per group were used in study and all experiments were
225 performed thrice.
226
227 **4. Results**
228 **4.1. *Tc_Wap*^{F} is exclusively expressed in adult female tissues**
229 *Tc_Wap*^{F} (XM_008200434.2) was identified in the sex-specific transcriptome of six-day old
230 adult *T. castaneum*. The *Tc_Wap*^{F} gene harbours 2 exons and 1 intron with an ORF of 240 bases
231 encoding 80 amino acids (aa) (Fig. S1A).
232 *Tc_Wap*^{F} was found to express only in female adults in our stage wise developmental profiling
233 (Fig. 1A). No expression of *Tc_Wap*^{F} was detected during larval or pupal stages. Further it was
234 found to express in the ovary, gut, and fatbody tissues of female adults (Fig. 1B). Females were
235 found to possesses 700-fold higher expression of *Tc_Wap*^{F} as compared to males in our qRT-

236 PCR analysis (Fig. 1C). Increasing expression pattern of *Tc_Wap*^{F} was observed in the
237 developing embryos (Fig. S3).
238
239 **4.2. Expression of *Tc_Wap*^{F} is not regulated by sex-determination cascade of *T. castaneum***
240 To analyze whether the sex-specific expression of *Tc_Wap*^{F} is regulated by the sex-
241 determination cascade of *T. castaneum*, we scanned the promoter sequence of *Tc_Wap*^{F} for the
242 presence of Doublesex (Dsx) binding site. The *dsx* (a transcription factor) is the most
243 downstream gene of insect sex determination cascade and is known to regulate the transcription
244 of sex-specifically expressed genes (Shukla and Palli, 2012b; Clough et al., 2014). A conserved
245 Dsx-binding motif (ACAATGT) was found about 4.5 Kb upstream to the *Tc_Wap*^{F} transcription
246 start site.
247 dsRNA (ds*Tcdsx*) injections (in male and female adults of *T. castaneum*) targeting common
248 region of *Tcdsx* led to a significant reduction in the gene expression in both male and female
249 (Fig. 2A). However, no change in the expression of *Tc_Wap*^{F} was observed in *Tcdsx* knockdown
250 adults (females or males) as compared to control adults (females or males) clearly indicating
251 that sex-specific expression of *Tc_Wap*^{F} is not regulated by *Tcdsx* (Fig. 2B).
252
253 We further examined if female-specific expression of *Tc_Wap*^{F} is regulated by some unknown
254 transcription factor (other than TcDsx) controlled by *TcTra*. TcTra is a splicing factor
255 responsible for female specific splicing of *Tcdsx* (Shukla and Palli, 2012b). Similar to the
256 strategy employed earlier dsRNA mediated knockdown of *Tctra* was done in females. *dsTctra*
257 injections led to an efficient knockdown of the target gene as evident by the detection of both,
258 female and male *Tctra* isoforms in *dsTctra* injected females (Shukla and Palli, 2014) in our RT-
259 PCR results (Fig. S4). Surprisingly, no change in the expression levels of *Tc_Wap*^{F} was
260 observed in *Tctra* knockdown females (Fig. 2C). Our results suggest that female-specific
261 expression of *Tc_Wap*^{F} is independent of the classical sex determination cascade of *T.*
262 *castaneum*.
263
264 **4.3. Development of eggs laid by *Tc_Wap*^{F} knockdown females is arrested**
265 dsRNA injections targeting *Tc_Wap*^{F} in females lead to a ~12-fold reduction in the expression
266 of *Tc_Wap*^{F} (Fig. 3A). Eggs laid by *Tc_Wap*^{F} knockdown females developed normally until 45
267 hours from their laying. These eggs showed underdeveloped germband, shrivelled and hollow
268 appearance of embryos in their later stages of development (Fig.3B). Further, none of the eggs
269 laid by *Tc_Wap*^{F} knockdown females hatch even after 96 hours whereas the eggs laid by

270 females injected with control dsRNA developed normally and hatched into larvae within 65-
271 72 hours of egg laying.

272

273 **4.4 Waprin is conserved across insecta**

274 A total of 65 Waprin and Waprin-related protein sequences from seven different orders of class
275 insecta and six outgroups were used for phylogenetic analysis. This list also included two
276 additional *waprin* genes (*Tc_Wap*^{2} and *Tc_Wap*^{3}) identified from the database of *T. castaneum*.
277 The tree highlighted that *Tc_Wap*^{F} and its homologs *Tc_Wap*^{2} and *Tc_Wap*^{3} started from the
278 same parent branch but diverged into different sister branches (Fig. 4A). Tc_Wap^{F} was found
279 to be most closely related to Waprin of *T. madens*, while Tc_Wap^{2} protein sequence was found
280 to be more similar to *Sandaracinus amylolyticus*. On the other hand, Tc_Wap^{3} protein sequence
281 showed similarity to the Waprin of *Hermetia illucens*.

282 Domain architecture analysis using TB tools predicted the presence of WAP domains in all the
283 protein sequences analysed (Fig. 4B). The WAP domain was found to be conserved in the
284 Waprin proteins of all the species included in our analysis. The number of these WAP domains
285 varied (ranging from one to four) in different Waprin proteins. Tc_Wap^{F} possesses a single
286 WAP domain whereas Tc_Wap^{3} has two WAP domains. Besides, some of the Waprin proteins
287 were found to possess additional domains also, predominantly PLAC and Kunitz domain.

288

289 **5. Discussion**

290 Waprin (WAP domain containing) proteins are found in diverse groups of organisms (Alam et
291 al., 2019) and are known to possess antiviral (Chen et al., 2008), antimicrobial (Ali et al., 2002;
292 Hagiwara et al., 2003; Hauton et al., 2006; Smith et al., 2008; Imjongjirak et al., 2009; Li et
293 al., 2013), Na-K ATPase inhibitory, protein and proteinase inhibitory (Ali et al., 2002; Li et al.,
294 2013) as well as immunomodulatory functions (Gao et al., 2021). In addition to their presence
295 in snake venom, Waprin proteins have also been reported in a few insect species, such as the
296 wood-boring woodwasp, *S. nitobei* (Gao et al., 2021), the guinea ant, *T. bicarinatum* (Bouzid
297 et al., 2014) and honeybees (*A. mellifera*) (Lee et al., 2023). Ironically, except for the
298 microbicidal and anti-elastolytic activities of Amwaprin, a recently discovered Waprin protein
299 from *A. mellifera*, the biological significance and evolutionary history of Waprin proteins in
300 the insect kingdom remain largely unknown (Lee et al., 2023).

301 Our study presents a detail analysis of *Tc_Wap*^{F}, a female-specific homologue of snake Waprin
302 in *Tribolium castaneum* including its unique expression patterns and crucial role in embryonic

303 development. *Tc_Wap*^{F}, which possesses eight conserved cysteine residues (Fig. S1B), a typical
304 feature of Waprin proteins is exclusively expressed in adult female tissues, including the ovary,
305 gut, and fatbody (Fig. 1B). Besides, we also identified two additional *waprin* genes (*Tc_Wap*^{2}
306 and *Tc_Wap*^{3}) in *Tribolium* (Fig. S2); however, these genes were found to express in a non-sex
307 specific manner (Fig. S2). Sexually dimorphic expression of *Tc_Wap*^{F} indicated its function in
308 female specific physiology or reproduction. To deduce the biological relevance of sex-specific
309 expression of *Tc_Wap*^{F}, dsRNA mediated knockdown experiments were performed in adult
310 females. Despite the significant knockdown in the expression of *Tc_Wap*^{F} (Fig. 3A), no
311 observable changes were noticed in the morphology or anatomy of the knockdown individuals
312 (data not shown). Furthermore, the fecundity of *Tc_Wap*^{F} knockdown females were unaffected
313 (data not shown). Interestingly, none of the eggs laid by *Tc_Wap*^{F} knockdown females hatch.
314 We hypothesized the failure of fertilisation or the growth arrest of fertilised embryos as the
315 reason for the same. To test this hypothesis, time course tracking of staged eggs either laid by
316 *Tc_Wap*^{F} knockdown females (knockdown eggs/embryos) or by control females (control
317 eggs/embryos) was done. Head and leg appendices became prominent in the control embryos
318 within 72 hours of their development whereas only a few larval structures were noticed in the
319 knockdown embryos (Fig. 3B). Growth of knockdown embryos appeared arrested at the
320 germband retraction stage and ultimately these eggs failed to hatch and started desiccating after
321 60-65 hours of egg laying. The expression of *Tc_Wap*^{F} in female adults (Fig. 1A), its gradual
322 increase in developing eggs (Fig. S3) and the failure of hatching of eggs laid by *Tc_Wap*^{F}
323 knockdown females points towards the crucial role of *Tc_Wap*^{F} in embryonic development in
324 *T. castaneum*.

325 Since the effect of *Tc_Wap*^{F} injections were pronounced in the embryos laid by injected
326 females, there could be two possibilities; 1) *Tc_Wap*^{F} transcript (or protein) may be maternally
327 supplied to the embryos for their proper development. Injections of ds*Tc_Wap*^{F} in the mother
328 lead to a reduced transfer of *Tc_Wap*^{F} transcript (or protein) in the embryos resulting in the
329 adverse effect on their development. This hypothesis is supported by the detection of *Tc_Wap*^{F}
330 transcripts in abundance during 0-2 hours of egg stage (unfertilised) and its subsequent absence
331 in the eggs of 2-4 hours development. *Tc_Wap*^{F} transcripts were again detected in 6-8 hours
332 old eggs which was found to be gradually increased in later stages (Fig. S3). The expression
333 profile of *Tc_Wap*^{F} during the embryonic development of *T. castaneum* suggests the switching
334 of *Tc_Wap*^{F} transcription from maternal to zygotic. 2) Alternatively, the dsRNA injected into
335 female adults might have been passed to the embryos, leading to a reduction of Tc_Wap^{F}

proteins in embryos and their growth arrest. In either of the cases, Tc_Wap^F seems to be essentially required for the development of *Tribolium* embryos.

Our hypothesis aligns with several previous studies demonstrating strategies adopted by mother to invest parental resources for the enhanced survival of their offspring. For example, in *Blatella germanica* the maternal supply of hydrocarbons is required to prevent the water loss from eggs (Fan et al., 2008). Deas *et al*. (2012), reported the plasticity in the egg laying behaviour of *Mimosestes amicus* (a seed beetle) female to protect its eggs from parasitic wasps (Deas and Hunter, 2012). Another evidence of maternal strategies for the enhanced survival of offsprings comes from mice, where high expression levels of *Wap* gene in the mammary glands of lactating mice play an essential role in nourishing their offspring (Triplett et al., 2005; Oftedal, 2012). Interestingly, the authors noticed a reduced embryonic development but failed to observe any phenotypic abnormalities in the mammary glands of *Wap*-knockout females which has striking resemblance to the results obtained from our knockdown studies.

Furthermore, the expression of *Tc_Wap^F* in the ovary and fatbody has a relevance as the ovary is the site of egg development, and the fatbody is the major site of vitellogenin synthesis, which facilitates yolk deposition in eggs (Fig. 1B). At present, we are unable to justify the reason for high expression of *Tc_Wap^F* in the *Tribolium* gut but a possible antimicrobial role of Tc_Wap^F similar to the case of other Waprin proteins, cannot be denied.

The central axis (*tra-dsx*) of insect sex determination cascade is also conserved in *T. castaneum* (Shukla and Palli, 2012a). Sex-specifically spliced *Tcdsx* produces sex-specific proteins, TcDsx^M in males and TcDsx^F in females, which are sex-specific transcription factors regulating all the genes expressed in a sex-specific manner (Shukla and Palli, 2012b). The expression levels of sex specific Dsx targets have been shown to increase in one sex while decrease in another sex upon Dsx knockdown in the two sexes (Verhulst and van de Zande, 2015). To explore whether the sex-specific expression of *Tc_Wap^F* gene is regulated by TcDsx^F, we examined the presence of TcDsx binding sites in the *Tc_Wap^F* gene. Although a conserved Dsx-binding motif (ACAATGT) was identified 4.5 Kbp upstream to the *Tc_Wap^F* transcription start site, we did not observe a decrease in the expression of *Tc_Wap^F* in *Tcdsx* knockdown females (Fig. 2B). Additionally, no upregulation in the expression of *Tc_Wap^F* was seen in *Tcdsx* knockdown males. We further hypothesized that *Tc_Wap^F* transcription might be regulated by an undiscovered transcription factor under the control of TcTra, a splicing regulator of *Tcdsx*. To test this hypothesis, we performed the knockdown of *Tctra* in female adults, but no change

368 in the expression *Tc_Wap*^{F} was observed in *Tctra* knockdown females (Fig. 2C). These results
369 suggest that the female-specific expression of *Tc_Wap*^{F} is not governed by the established sex
370 determination cascade of *T. castaneum*. It is possible that the sex-specific expression of
371 *Tc_Wap*^{F} is regulated by a pathway diverging upstream of *Tctra* (Shukla and Palli, 2012a;
372 Netschitailo et al., 2023). Alternatively, the sex-specific expression of *Tc_Wap*^{F} gene could
373 result from differences in chromatin confirmation between the sexes. This is supported by a
374 recent study highlighting the differential chromatin confirmation as the regulator of sex-biased
375 gene expression in two *Drosophila* species, *D. melanogaster* and *D. simulans* (Nanni et al.,
376 2023).

377 In conclusion, we identified a sex-specific homologue of snake *Waprin* (*Tc_Wap*^{F}) from *T.*
378 *castaneum* and analysed its function. Our study suggested the crucial role of *Tc_Wap*^{F} in the
379 embryogenesis of *T. castaneum* which provides the foundation for further investigations into
380 the molecular functions of *waprin* genes in other insects. The sex-specific expression of
381 *Tc_Wap*^{F} is not governed by the classical sex-determination cascade of *T. castaneum*. *waprin*
382 genes were found to be conserved across insect species which indicates its functional integrity
383 throughout evolution. Future research focusing sex-specific *waprin* genes and its regulation in
384 insects could provide valuable insights into their potential applications in enhancing the insect
385 control strategies utilising sterile insect technology.

386

387 **6. Author's Contribution:**

388 CC and JNS conceived the idea of manuscript. CC performed knockdown, PCR and qRT-PCR
389 experiments. DK did the phylogenetic analysis. KKM maintained the insect cultures and helped
390 CC in preparation of the manuscript. CC, VV and JNS wrote the manuscript and all others
391 agreed to it. All authors contributed to the article and approved the submitted version.

392

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528

529

530 **Figure Legends:**

531 **Fig.1A:** Developmental expression profiling of *Tc_Wap*^{F} of *T. castaneum*. RT-PCR analysis
532 was performed using stage specific cDNAs (6th Instar Larvae, Q Stage, Larvae, female pupae,
533 male pupae, female adults and male adults) as templates. **B)** Tissue (ovary, gut, head and
534 fatbody of female adults) specific expression profile of *Tc_Wap*^{F}. **C)** Relative expression of
535 *Tc_Wap*^{F} in 6-day old adult male and female. The error bars represent the standard deviation
536 (**p value=0.0035).

537 **Fig.2A:** Graphical representation of the knockdown efficiency of *Tcdsx*. ~ 6-fold and 9-fold
538 reduction was observed in *Tcdsx* expression in female and males respectively. Fold change (*p
539 value≤ 0.0311; **p value ≤ 0.0003). **B)** The relative expression of *Tc_Wap*^{F} was quantified in
540 response to *Tcdsx* knockdown in female and male individuals. Error bars represent standard
541 deviation. **C)** Graphical representation of *Tc_Wap*^{F} expression upon *TcTra* knockdown. The
542 knockdown efficiency was analysed on 5th day after knockdown. Five biological replicates
543 were used in experiment.

544 **Fig.3A:** Graphical representation of the relative expression of *Tc_Wap*^{F} in the knockdown and
545 control females. Three biological replicates of both control and knockdown individuals were
546 taken for analysis. Error bars represent standard deviation (**p value≤ 0.0020). **B)** Microscopic
547 images of different stages of *Tribolium* embryos laid by the ds*GFP* and ds*Tc_Wap*^{F} injected
548 females. Eggs laid by ds*Tc_Wap*^{F} injected females developed normally until 45 hours, but
549 uneven germband was formed subsequently. White arrow represents the region of abnormality.
550 Embryos were stained by Hoechst dye and imaged using fluorescence microscope (Scale bar
551 100µm, magnification 10X).

552 **Fig.4A:** Circular phylogenetic tree of WAP proteins from different species. The protein
553 sequences were aligned using MEGA11 and the tree was constructed using the neighbour-

joining method. **B)** Domain architecture of various WAP domain containing proteins across different species. Different domains are colour coded.

Fig.S1A: Schematics representing the structures of different *waprin* genes (*Tc_Wap*^{F}*, Tc_Wap*^{2} *and Tc_Wap*^{3}) of *T.castaneum. Tc_Wap*^{F} consists of two exons and one intron, *Tc_Wap*^{2} contains three exons and two introns whereas *Tc_Wap*^{3} possess two exons and one intron. Boxes represent exons and lines represent the introns. Numbers represent the length of respective exon or intron. Start and stop codons are represented by green and red bars (vertical). Horizontal arrows represent the primer positions; orange- dsRNA primers, black- semi quantitative PCR; pink- qRT PCR. **B)** Sequence alignment of *Tribolium* Waprin proteins. Conserved cysteine residues are shown in yellow boxes.

Fig. S2: Expression profiling of *Tc_Wap*^{2} and *Tc_Wap*^{3}. *Tc_RPS-18* was used as an endogenous control.

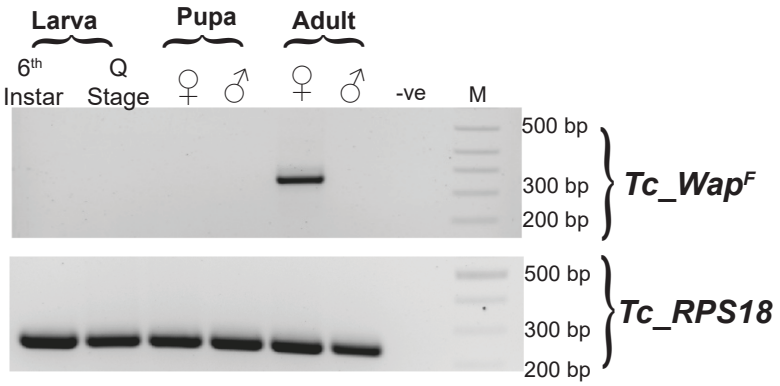
Fig.S3: Expression profile of *Tc_Wap*^{F} in staged embryos. The time mentioned in the figure represents the stage of eggs used for RNA isolation. *Tc_RPS-18* gene was used as an endogenous control.

Fig.S4: Gel image showing the splicing status of *Tctra* in control (*dsGFP* injected) and knockdown (ds*Tctra* injected) female adults. RT-PCR was performed using cDNA synthesised from RNA isolated from control females (C1-C4) and knockdown females (T1-T4).

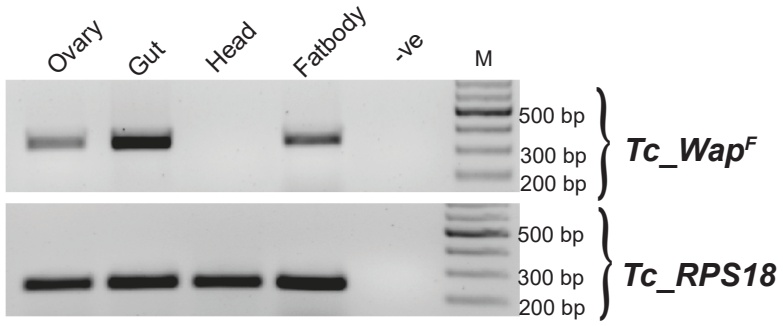
Table S1: List of primers (and their details) used in the study.

Table S2: *waprin* genes of *T.castaneum* and their respective accession ID's.

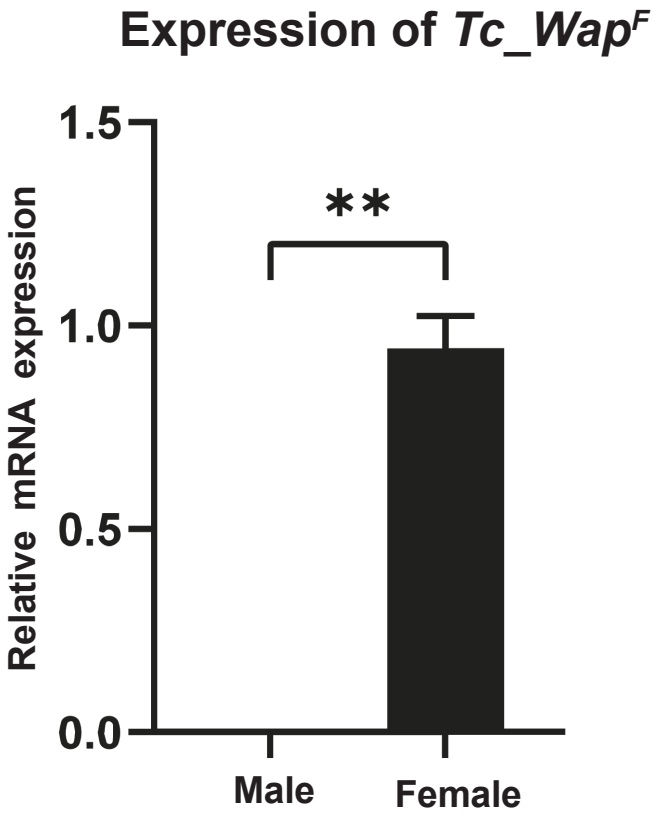
A)

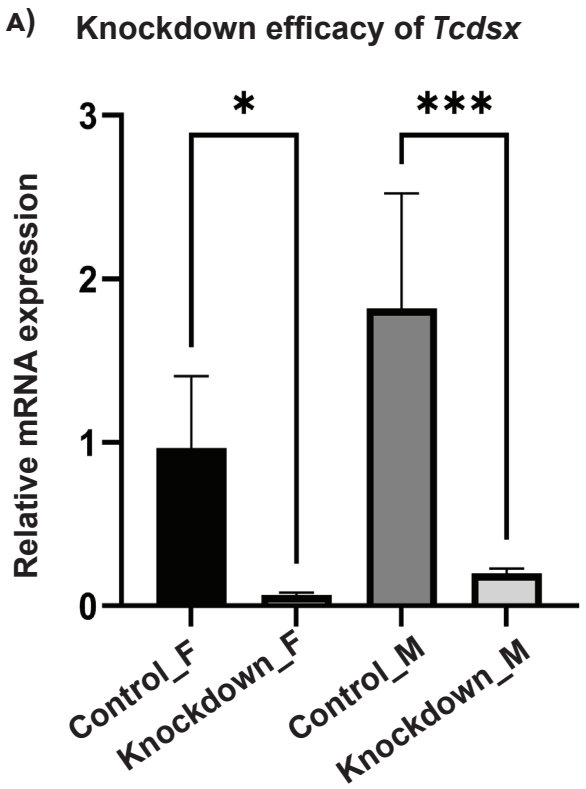

Larva
Pupa
Adult
6th Instar
Q Stage
♀ ♂
♀ ♂
-ve
M
500 bp
300 bp
200 bp
Tc_WapF
500 bp
300 bp
200 bp
Tc_RPS18

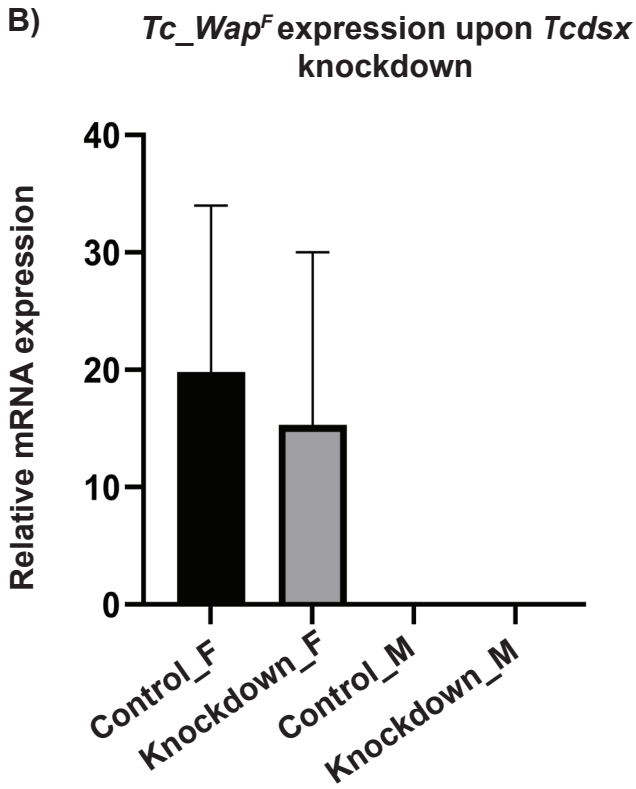
B)

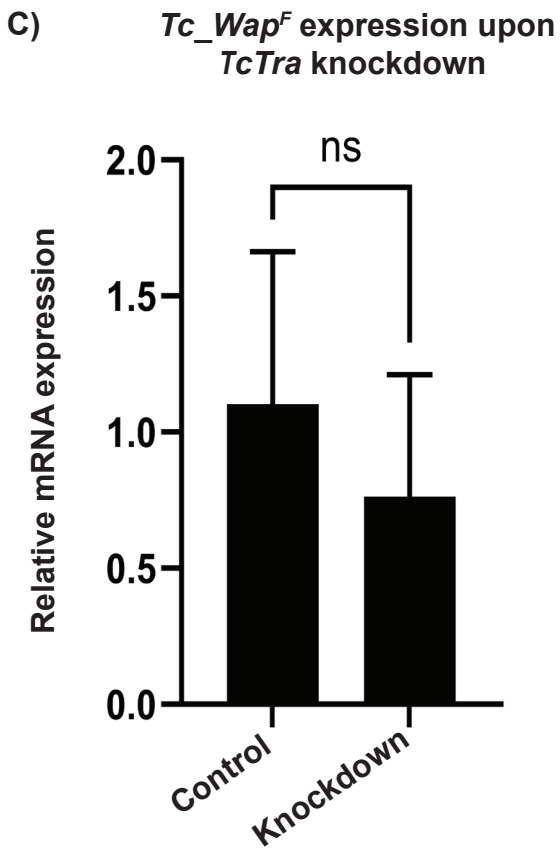

Ovary
Gut
Head
Fatbody
-ve
M
500 bp
300 bp
200 bp
Tc_WapF
500 bp
300 bp
200 bp
Tc_RPS18

C)

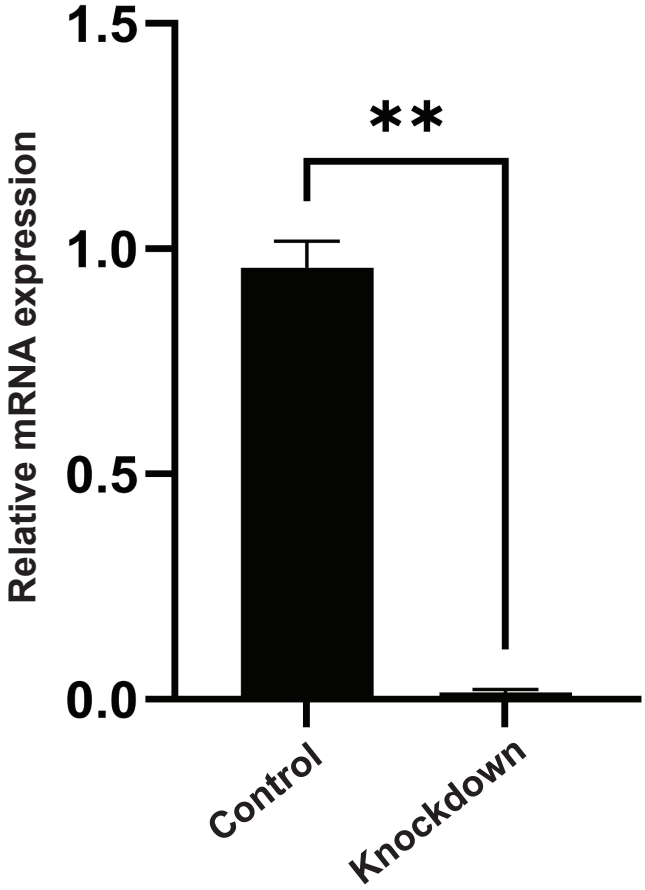

Expression of Tc_WapF
Relative mRNA expression
1.5
1.0
0.5
0.0
**
Male
Female

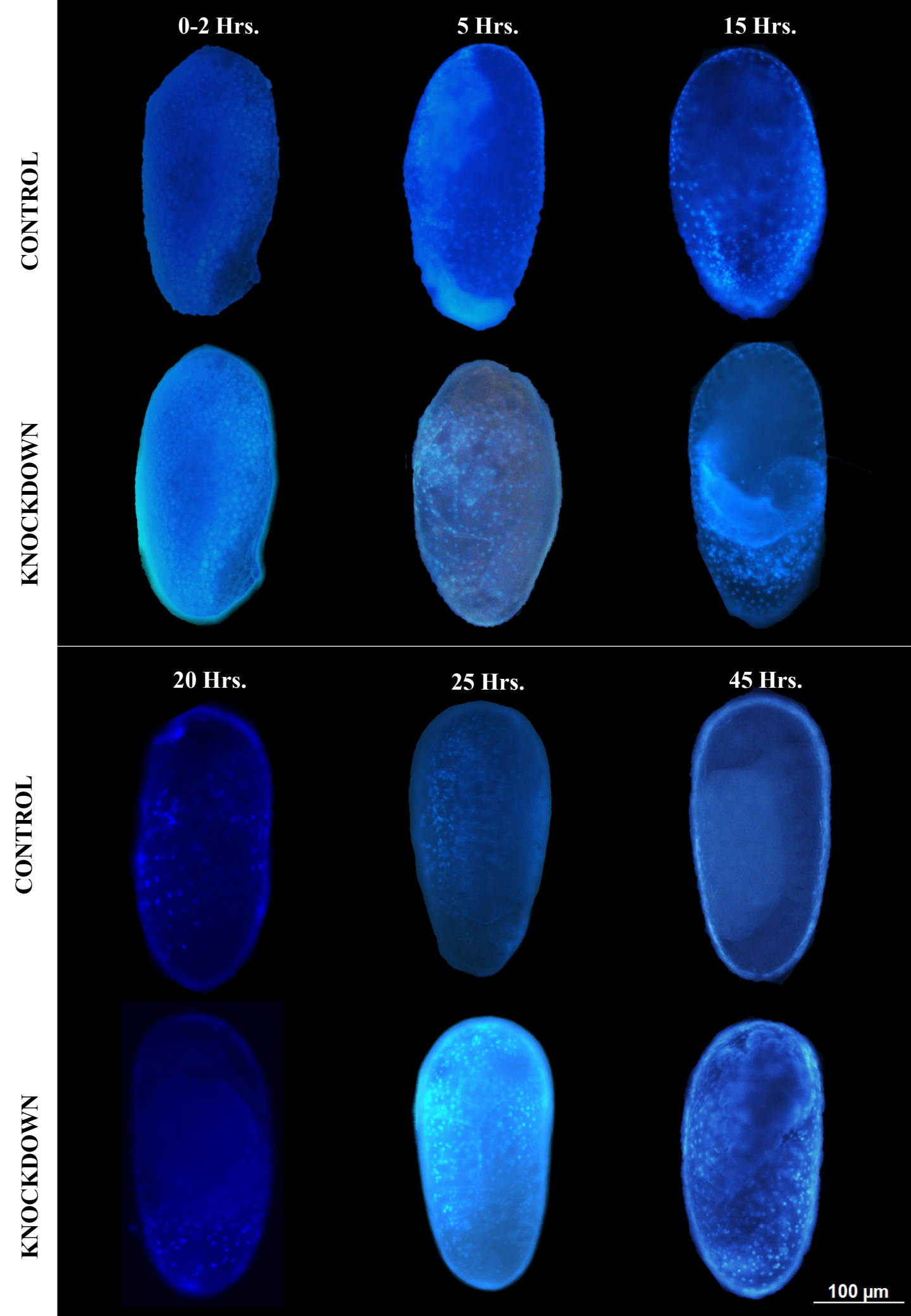

A)
Knockdown efficacy of Tcdsx
Relative mRNA expression
3
2
1
0
*
***
Control_F
Knockdown_F
Control_M
Knockdown_M

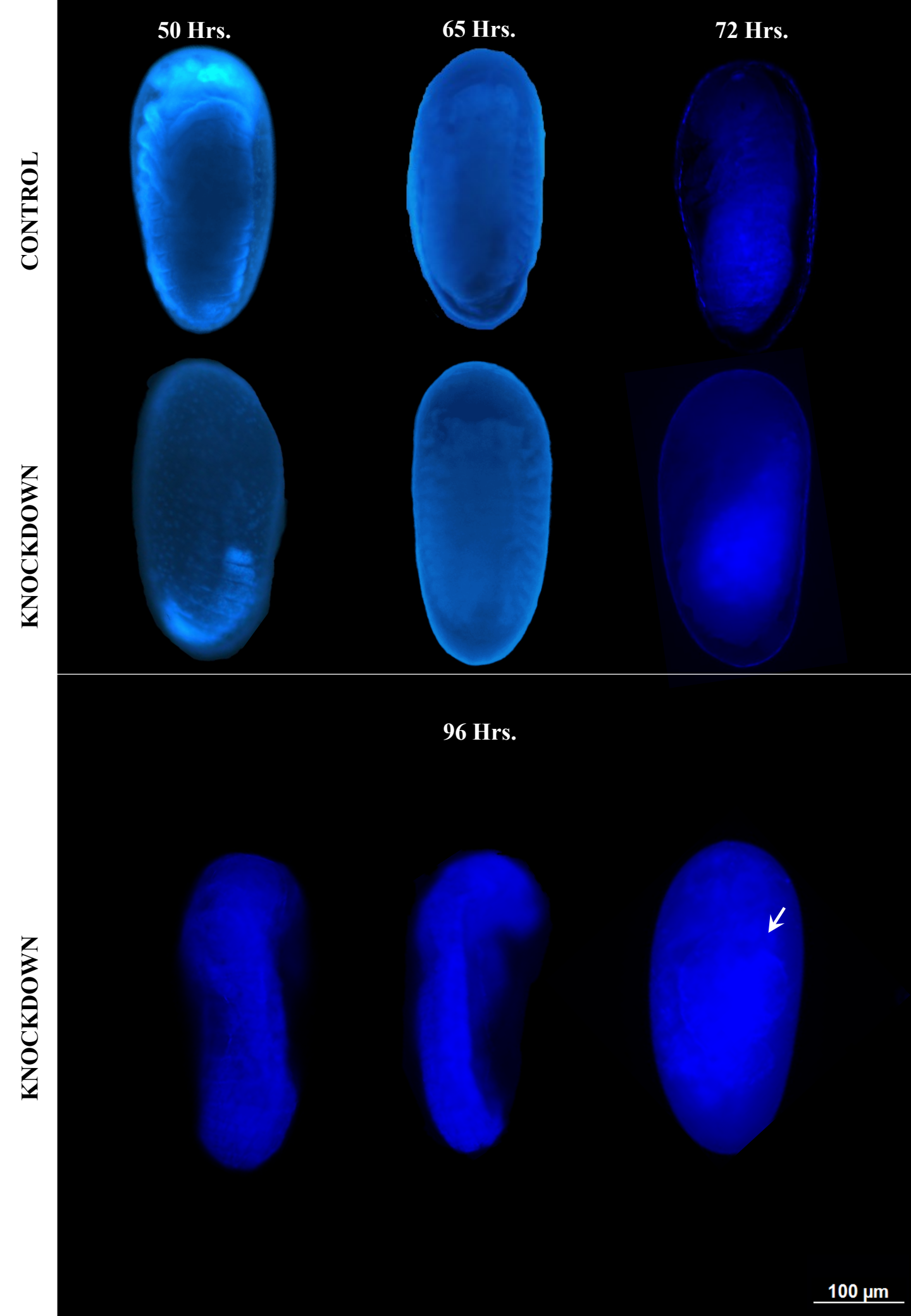

B)
Tc_WapF expression upon Tcdsx knockdown
Relative mRNA expression
40
30
20
10
0
Control_F
Knockdown_F
Control_M
Knockdown_M

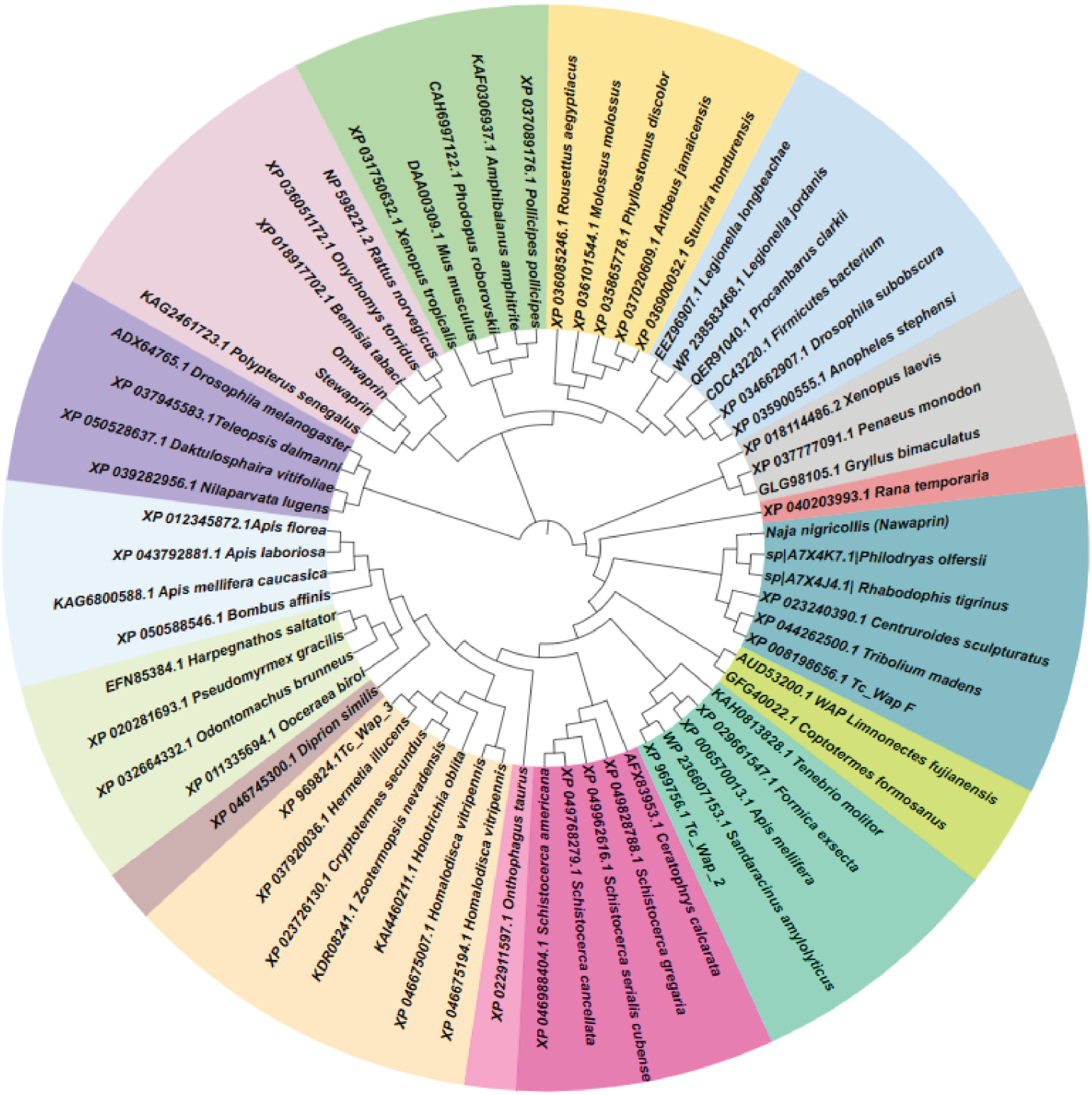

C)
Tc_WapF expression upon TcTra knockdown
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
ns
Control
Knockdown

A) Knockdown efficiency of *Tc_Wap*^{F}




0-2 Hrs.
5 Hrs.
15 Hrs.
CONTROL
KNOCKDOWN
20 Hrs.
25 Hrs.
45 Hrs.
CONTROL
KNOCKDOWN
100 µm


50 Hrs.
65 Hrs.
72 Hrs.
CONTROL
KNOCKDOWN
96 Hrs.
KNOCKDOWN
100 µm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XP 036101544.1 Molossus molossus
XP 035865778.1 Phyllostomus discolor
XP 037020609.1 Artibeus jamaicensis
XP 036900052.1 Sturnira hondurensis
EEZ96907.1 Legionella longbeachae
WP 238583468.1 Legionella jordanis
QER91040.1 Procambarus clarkii
CDC43220.1 Firmicutes bacterium
XP 034662907.1 Drosophila subobscura
XP 035900555.1 Anopheles stephensi
XP 018114486.2 Xenopus laevis
XP 037777091.1 Penaeus monodon
GLG98105.1 Gryllus bimaculatus
XP 040203993.1 Rana temporaria
Naja nigricollis (Nawaprin)
sp|A7X4K7.1|Philodryas olfersii
sp|A7X4J4.1| Rhabodophis tigrinus
XP 023240390.1 Centruroides sculpturatus
XP 044262500.1 Tribolium madens
XP 008198656.1 Tc_Wap F
AUD53200.1 WAP Limnonectes fujianensis
GFG40022.1 Coptotermes formosanus
KAH0813828.1 Tenebrio molitor
XP 029661547.1 Formica exsecta
XP 006570013.1 Apis mellifera
WP 236607153.1 Sandaracinus amylolyticus
XP 969756.1 Tc_Wap_2
AFX83953.1 Ceratophrys calcarata
XP 049828788.1 Schistocerca gregaria
XP 049962616.1 Schistocerca serialis cubense
XP 049768279.1 Schistocerca cancellata
XP 046988404.1 Schistocerca americana
XP 022911597.1 Onthophagus taurus
XP 046675194.1 Homalodisca vitripennis
XP 046675007.1 Homalodisca vitripennis
KAI4460211.1 Holotrichia oblita
KDR08241.1 Zootermopsis nevadensis
XP 023726130.1 Cryptotermes secundus
XP 037920036.1 Hermetia illucens
XP 969824.1Tc_Wap_3
XP 046745300.1 Diprion similis
XP 011335694.1 Ooceraea biroi
XP 032664332.1 Odontomachus brunneus
XP 020281693.1 Pseudomyrmex gracilis
EFN85384.1 Harpegnathos saltator
XP 050588546.1 Bombus affinis
KAG6800588.1 Apis mellifera caucasica
XP 043792881.1 Apis laboriosa
XP 012345872.1Apis florea
XP 039282956.1 Nilaparvata lugens
XP 050528637.1 Daktulosphaira vitifoliae
XP 037945583.1Teleopsis dalmanni
ADX64765.1 Drosophila melanogaster
KAG2461723.1 Polypterus senegalus
Stewaprin
Omwaprin
XP 018917702.1 Bemisia tabaci
XP 036051172.1 Onychomys torridus
NP 598221.2 Rattus norvegicus
XP 031750632.1 Xenopus tropicalis
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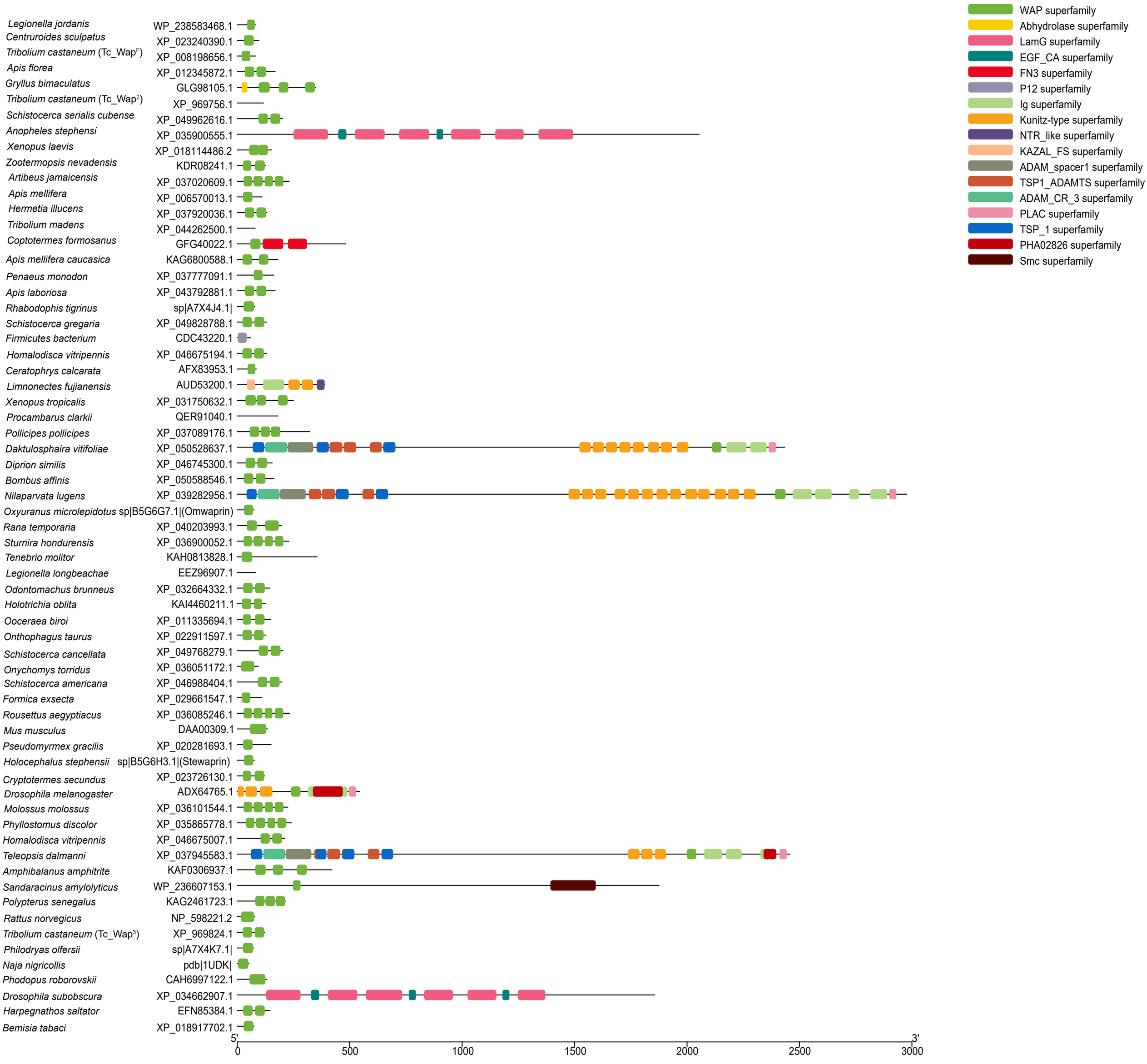
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Gryllus bimaculatus GLG98105.1
Tribolium castaneum (Tc_Wap[2]) XP_969756.1
Schistocerca serialis cubense XP_049962616.1
Anopheles stephensi XP_035900555.1
Xenopus laevis XP_018114486.2
Zootermopsis nevadensis KDR08241.1
Artibeus jamaicensis XP_037020609.1
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_Wap[3]) 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'
3'
0
500
1000
1500
2000
2500
3000
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