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The effect of *Tep1* knockdown on parasitoid resistance in *Drosophila melanogaster*

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Abstract

Parasitoids are parasites that lay their eggs inside a host and have to kill this host in order to develop successfully. They can have a profound effects on the host populations. The interaction between parasitoids and their host can be seen as co-evolution, which can lead to a metaphorical arms race. Drosophila relies on its innate immune system to survive parasitoid attacks. By using blood cells to encapsulate and killing parasitoid eggs it finds a way to escape certain death. The genetic basis of this response is not yet fully understood, though the thioester-containing protein (Tep) gene family is thought to play an important role. Especially the function of *Tep1* remains to be elucidated. This gene is found to possess significant sequence variation and to be under strong positive selection in natural populations. A knock-down of *Tep1* was attempted to assess its importance in the encapsulation response. Potential redundancy and an incorporation of its role by the closely related *Tep2* was also checked. Some complications arose with regards to the effectiveness of the knock-down and the melanization ability of the larvae, but no hard evidence was found to link the *Tep1* expression with differences in encapsulation success. Expressions levels of *Tep2* did not indicate redundancy of *Tep1*, although the expression of both genes was strongly and positively correlated. Furthermore, the external temperature, humidity and air pressure explained a significant part of the variation in melanization success. This illustrates the variability of the assumed to be stable environment in a lab and should be considered in future research.

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Introduction

Insects account for the great majority of animal species on earth, showing an incredible diversity (Grimaldi and Engel 2005). They play an essential role in ubiquitous interactions, which can be based on mutualism or commensalism, but does not rarely end up in parasitism (Samways et al. 2010). Parasitoids are a special type of parasite which kill their hosts in order to develop successfully (Lafferty and Kuris 2002). They usually are insects with a free-living adult stage that require a host for egg and juvenile development (Carton et al. 1986; Schmid-Hempel 2011). Host-parasitoid interactions can have a profound effect on the host population, as parasitoids are thought to cause a considerable part of the mortality for a variety of insects species (Quicke 1997; Strand and Pech 1995). Parasitoids can therefore be of an enormous ecological and economic importance as a successful biological agent of pests (LaSalle and Gauld 1993). First described by Van Valen (1973), the dynamics between a parasitoid and its host can be seen as a metaphorical arms race. Parasitoids and their hosts often co-evolve, as a result of the selection pressure they exert on each other (Schmid-Hempel 2011). This co-evolutionary arms race has been fairly well characterized for *Drosophila* (Diptera: Drosophilidae) and various of its larval parasitoids (Carton et al. 1986; Fleury et al. 2009).

Insect immune system

Other than vertebrates, invertebrates lack an adaptive immune system and thus depend on their innate immune system to cope with parasitoid attacks. This immune system can be divided into a cellular and a humoral component (Lagueux et al. 2000; Meister and Lagueux 2003). Humoral immunity is triggered by a bacterial or fungal infection, leading to a massive production of antimicrobial peptides and melanin (Lemaitre and Hoffmann 2007; Meister and Lagueux 2003). The cellular immunity comprises a hemocyte-mediated response. Hemocytes (blood cells) differentiate into cells with different functions, including pathogen recognition, phagocytosis or encapsulation (Lavine and Strand 2002). Encapsulation is the process of the attachment of hemocytes to foreign matter, as parasitoid eggs, which eventually leads to the forming of a capsule and neutralization of the pathogen (Pech and Strand 1996). Comparison of hemocytes between species is intrinsically complex, as morphologically similar cells often perform different functional roles (Pech and Strand 1996). In *Drosophila* three different types of hemocytes have been identified. The lamellocytes are flat adhesive cells that are only produced after parasitization and are involved in the encapsulation of foreign matter too large to be phagocytized (Lavine and Strand 2002; Ribeiro and Brehélin 2006; Rizki and Rizki 1980). Plasmatocytes, on the other hand, are the main phagocytes in the hemocoel. Besides phagocytizing, these cells synthesize and secrete antimicrobial peptides and are also involved in the encapsulation process (Agaisse et al. 2003; Williams 2007). Melanization of the capsule is mediated by the third type of hemocytes, the crystal cells, that contain phenoloxidases, synthesize melanin and generate cytotoxic free radicals which aid in neutralizing invading pathogens (Fauvarque and Williams 2011; Pech and Strand 1996; Ribeiro and Brehélin 2006; Williams 2007).

Drosophila and its parasitoids

To successfully complete their life cycle parasitoids have to circumvent the immune system of their host to avoid encapsulation. The parasitoid has to prevent elimination either by evading the local immune system (e.g. by making the egg inaccessible for hemocytes, molecular mimicry or local inactivation of the immune system) or by active immune suppression during oviposition (Lemaitre and Hoffmann 2007; Schmid-Hempel 2011).

The parasitoid wasp *Asobara tabida* (Hymenoptera: Braconidae) uses passive evasion to prevent elimination by its host, species of the fruit fly genus *Drosophila*. Its eggs possess a sticky chorion, which becomes easily attached to, and embedded in, the tissue of the fly larvae, rendering the hemocytes incapable of neutralizing the threat (Eslin and Prévost 2000; Kraaijeveld and van Alphen 1994; Lemaitre and Hoffmann 2007). Active immune suppression is used by cynipid parasitoid wasps of the genus *Leptopilina* (Hymenoptera: Figitidae). Two of the well-known species of this genes (*L.heterotoma* and *L.boulardi*) have been found to produce virus-like particles (VLPs) which suppress the hosts' immune system by destroying the lamellocytes. Although found to be less virulent, female *L. victoriae* inject a cocktail of VLPs in the host during oviposition as well (Morales et al. 2005; Prévost 2009).

Only species of the *melanogaster* subgroup (*D. erecta, D. melanogaster, D. sechellia, D. simulans* and *D. yakuba*), except *D. sechellia*, have the ability to encapsulate parasitoid eggs with lamellocytes (Salazar-Jaramillo et al. 2014). Besides this between-species variation in parasitoid resistance, also within *Drosophila* species the ability to successfully encapsulate a parasitoid egg varies. The ability of the various *Drosophila* populations to encapsulate these sticky eggs has been found to vary geographically in natural populations (Gerritsma et al. 2013). Furthermore, *Drosophila* resistance is negatively correlated with the stickiness of a parasitoid egg, as the sticky chorion hinders blood cells in forming a completely enclosed capsule around the egg (Kraaijeveld and van Alphen 1994). Eslin and Prevost (1996) have demonstrated the importance of the hemocyte concentration on parasitoid resistance, as *Drosophila* spp. carrying more blood cells in general had higher success in their defense reaction.

Genetic basis of the *Drosophila* immune response against parasitoids

The genetic basis of the encapsulation response of *Drosophila* to parasitoid eggs is not fully understood, although it is clear that many genes are involved in the immune response (Jalvingh et al. 2014; Schlenke et al. 2007; Wertheim et al. 2005). A large subset of these immunity genes show high levels of diversity or signs of adaptive evolution, particularly the genes encoding immune receptors, a family of receptors which facilitate binding pathogens (Obbard et al. 2009; Salazar-Jaramillo et al., 2014, in preparation). A similar pattern can be found in a family of well-known immune receptors in vertebrates, the major histocompatibility complex (MHC). MHC possesses regions of enormous diversity, reflecting the strong advantage of heterozygotes in the ability of the immune system to deal with an array of pathogens (Abbas et al. 2012; Hughes and Nei 1988). Positive selection on heterozygotes has thus maintained and even increased the diversity over time (Maruyama and Nei 1981).

Tep gene family

One of the receptor genes that has been shown to evolve under positive selection in Drosophila is *Tep1*. The *Tep1* gene is part of the Thioester-containing protein (*Tep*) family, a family of immune receptors. Genes from this family have been described in many different organisms such as nematodes, mammals, birds and insects. In vertebrate systems, the *Tep* genes encode for proteins which are essential components of the immune system, as complement factors C3, C4 and C5, which bind pathogens and act as opsonins, and as the protease inhibiting α_2-macroglobulins (Blandin and Levashina 2004). In insects this gene family has mostly been studied in *Drosophila* and mosquitos (Aoun et al. 2011; Blandin and Levashina 2004; Jiggins and Kim 2006; Little and Cobbe 2005). Six different *Tep* genes have been identified in the genus *Drosophila*, all of which are located on the 2L chromosome arm (Jiggins and Kim 2006; Lagueux et al. 2000). Four of these *Tep* genes (*Tep1-4*) contain a highly conserved region with a thioester motif and a hypervariable region consisting of roughly 60 residues (Lagueux et al. 2000; Stroschein-Stevenson et al. 2006). The hypervariable region of *Tep2* has multiple exons, allowing different transcripts with alternative splicing (Lagueux et al. 2000). Only three of the *Tep* genes, *Tep1*, *Tep2* and *Tep4*, are upregulated in case of an immune challenge against both micro- and macroparasites and are thus assumed to play a role in the innate immune system (Lagueux et al. 2000; Wertheim et al. 2005; Aoun et al. 2011; Salazar-Jaramillo et al. in preparation). While *Tep2* is found to be required for efficient phagocytosis of the bacteria *E. coli* (Stroschein-Stevenson et al. 2006), the exact functions of *Tep1* and *Tep4* still remain unclear.

A considerable amount of research has been done on the *Tep1* gene. This lead to the findings that the gene arose from a recent duplication and is one of the fastest evolving genes in *Drosophila* (Christophides et al. 2002; Jiggins and Kim 2006). It evolves under strong positive selection and has substantial variation and polymorphism levels. Both positive selection and the high heterozygosity levels could imply some co-evolutionary past between *Drosophila* and its parasites (Jiggins and Kim 2006; Salazar-Jaramillo et al. 2014). In the larvae, expression of *Tep1* is highest in the fat body, the hemocytes and the lymph glands (Aoun et al. 2011), while in adults other *Tep*s are more prevalent (Lagueux et al. 2000; Meister and Lagueux 2003). The expression of *Tep1* is not controlled by the Toll and imd signaling pathways, which regulate the majority of the immune system, but instead is strongly dependent on the Janus kinase (JAK) *Hopscotch* (De Gregorio et al. 2002; Lagueux et al. 2000). Interestingly, a deletion of four exons is located in the *Tep1* gene of *D. sechellia*, the only species of the *melanogaster* subgroup unable to encapsulate (Salazar-Jaramillo et al. 2014). Among three *Drosophila* spp. extensive differences in the level and speed of expression of this gene were found (Salazar-Jaramillo et al. 2014).

Gal4-UAS system

One commonly used method to functionally characterize the role of particular genes is through gene silencing. The GAL4/UAS system is frequently used in gene expression research (Traven et al. 2006). This system consists of a GAL4 regulatory element and an Upstream Activating Sequence (UAS) in

two separate transgenic lines. The GAL4 element, originally from the yeast *Saccharomyces cerevisiae*, is an 881-amino-acid protein (Lohr et al. 1995). It binds to a target UAS sequence of 5′-CGG-N11-CCG-3′ and activates transcription by recruiting the transcription machinery and coactivators (Traven et al. 2006). When combined by crossing the transgenic lines, the GAL4 thus activates transcription of the UAS element (Brand and Perrimon 1993; Duffy 2002). In gene silencing studies, the UAS element is followed by an inverted repeat sequence complementary to sequence of the gene of interest. This inverted repeat sequence forms double-stranded hairpin RNA molecules, which are processed into siRNAs. These small interfering RNAs promote degradation of the target RNA by the endogenous repair mechanisms of the cell, resulting in the silencing or knock-down of gene expression of *Tep1*.

Aims

The aim of this experiment was to clarify the function of the *Tep1* gene in cellular immunity, specifically in parasitoid resistance of *D. melanogaster*. Ultimately, this would enhance our understanding of the cellular immune response against parasitoid attacks and give insight in the co-evolutionary dynamics of host-parasitoid interactions. Using a GAL4-UAS knock-down construct, the expression of the *Tep1* gene was inhibited. The effect of the knock-down of *Tep1* on parasitoid resistance was determined by assessing the encapsulation ability of both *A. tabida* and *L. victoriae* parasitoid eggs. In preliminary studies, knock-down of *Tep1* decreased the encapsulation rate and increased the lamellocyte count (Salazar-Jaramillo et al. in preparation). Due to ambiguous results in the *Tep1* knock-down, additional experiments were necessary to validate the results of this study. Previous work also hypothesized that compensation by other *Tep* proteins may occur in case of a *Tep1* knock-down, as the gene was not strictly required to withstand bacterial or fungal infections (Aoun et al. 2011). Therefore the expression of the closely related gene, *Tep2*, was assessed as well. We expected that *Tep1* knock-down flies would show a reduced ability to encapsulate parasitoid eggs.

Methods

Insects

Laboratory lines of *D. melanogaster* from a w_{1118} background were used. The lines were kept in mass cultures at room temperature. Food was provided *ad libitum* in the form of enriched medium, containing agar, glucose, sucrose, yeast, cornmeal, wheat germ, soy flour, molasses, propionic acid and Tegosept.

As previous experiments using a single UAS *Tep1* construct had mixed success in knocking down *Tep1* expression (Salazar-Jaramillo et al. in preparation), we used females with a double UAS *Tep1* inverted repeat construct, each located on a different chromosome. These females carrying the UAS-*Tep1* constructs were crossed with DaGAL4 males. DaGAL4 is expressed in the entire body of *Drosophila*, implying that Tep1 expression would be knocked-down in all tissues. As controls, both the GAL4 line and the double UAS *Tep1* construct were crossed with the w_{1118} strain.

A second GAL4 driver, cgGAL4, was implemented in a more resistant genetic background (see *Generation cgGAL4 lines*). The cgGAL4 is expressed in the hemocytes and the fat body of the larvae. Females carrying this cgGAL4 construct were crossed with males from the UAS-*Tep1* line to generate a knock-down of the *Tep1* gene. As controls, the cgGAL4 flies were crossed with a w_{1118} strain and the UAS-*Tep1* flies with flies from Gotheron.

The parasitoid wasp *A. tabida* strain TMS was established as an isofemale line in 2010 from a cross between lines from Sospel (France) and Pisa (Italy) and is a moderately virulent wasp strain. The *Leptopilina victoriae* line was donated by the CNRS (Gif-sur-Yvette, France) and originated in Africa. Adults of both wasp species were kept at 12°C, a humidity of 50% and a light:dark regime of 12:12. Honey was provided as food source. *A. tabida* was reared at 20°C and *L. victoriae* at 25°C, both on second instar larvae of a low-resistant *D. melanogaster* strain.

Parasitization and resistance assay

The activity of the GAL4 construct is minimal at 18°C and optimal at 29°C. *A. tabida* has an optimal temperature of 20°C. Previous experiments have shown a single UAS *Tep1* construct not to be efficient at this temperature. The double UAS *Tep1*, on the other hand, was found to be effective in knocking down the *Tep1* gene at this temperature (see Results section). Experiments with the *A. tabida* TMS strain were therefore conducted at 20°C. Having a broader temperature range, the *L. victoriae* experiments were conducted at 25°C.

A single *A. tabida* parasitoid wasp was introduced to second instar *D. melanogaster* larvae. Upon successful parasitization, which is quantified as an injection of the ovipositor of the wasp in the larva for at least ten seconds, the larva was collected. The experiment was repeated on three days, with a sample size of 18-46 larvae per day and a total of 26-36 larvae per cross. As *L. victoriae* is reluctant to parasitize while being handled, three of these wasps were introduced to a petri dish containing fifty *Drosophila* larvae for three hours. Replicates were done on four days, with a sample size of 28-159

larvae per day and a total of 66-80 larvae per cross. Rates of parasitization were verified afterwards and controlled for in the analyses by adding this variable in the statistical models.

The resistance of *D. melanogaster* against parasitoid wasps was assessed by the encapsulation success. The parasitoid egg was dissected out of the larvae two days post-parasitization and the encapsulation ability was scored as the percentage of the total egg that is melanized. Encapsulation was considered as either successful, in case of a complete melanization, or unsuccessful, in case of an incomplete melanization. In the event of superparasitization, successful melanization was defined as a minimum of one egg completely melanized.

Tep1 and Tep2 expression

To check the efficiency of the knock-down of *Tep1* and the effect this had on *Tep2*, real-time qPCR was performed on both parasitized and control larvae for each of the crosses. RNA was extracted from three to six pooled parasitized larvae and a total number of biological replicates of 2-3 (*A. tabida* experiment), 8-12 (*L. victoriae* DaGAL4 experiment) and 5-9 (*L. victoriae* cgGAL4 experiment) per cross 24 hours after parasitization. The RNA extraction was done using Trizol (Invitrogen, Carlsbad, CA, USA) and the RNeasy plus mini kit (Qiagen, Hilden, Germany). Previous research has shown this time to be optimal as *Tep1* expression levels is highest 12-24 hours after the immune challenge (Wertheim et al. 2005).

The purity of the extracted RNA was checked using Nanodrop (Thermo scientific) and cDNA was synthesized from the RNA using RevertAid Reverse Transcriptase (Thermo scientific). The real-time qPCR was conducted using an Applied Biosystems 7300 Real Time PCR system, using ABsolute™ QPCR SYBR® Green ROX mix (Thermo scientific) and the following primers, designed by Salazar-Jaramillo et al. (in preparation) using PerlPrimer v1.1.21 (Marshall 2004): *Tep1*-mRNA *forward* AGTCCCATAAAGGCCGACTGA and *reverse* CACCTGCATCAAAGCCATATTG. As endogenous references, the housekeeping genes Forkhead domain 68A (fd68A) and α-Tubulin at 84B (αtub84B) were used. Primers for these genes were respectively *FD68A forward* GCTAGTCCACGTCAGGGT, *reverse* GTCTGGAACAGATCCTGT, *αtub84B forward* GTTTGTCAAGCCTCATAGCC and *reverse* TGGATAGAGATACATTCACGCA (Salazar-Jaramillo et al. in preparation). *Tep2* activity was assessed using the following primers designed with PerlPrimer v1.1.21: *Tep2b forward* CTTCGAGAATCATGAACTGATCCC and *reverse* GCTCTCCAGCTTAGCAATGAC (Marshall 2004).

The real-time qPCR method is quantitative, which enables detecting the amplified cDNA as the reaction progresses. To detect the expression level for the genes of interest a fluorescent dye, SYBR Green I, is used. This dye fluoresces when bound to double-stranded DNA and allows for relative quantification (i.e. comparison of expression among different samples). An increasing fluorescence can thus be seen with increasing production of double-stranded DNA for the targeted gene. The fluorescence is a measure for the amount of mRNA and hence the activity of the specific gene.

To analyze these qPCR data, the initial concentration (N_0) was determined for each sample using Linreg (Ramakers et al. 2003). This program uses individual PCR efficiencies for each sample and a window-of-linearity approach to deduce the starting mRNA concentration. The average of two technical replicates was obtained and this value was standardized with the geometric mean of the two reference genes, and then compared among the different groups and treatments.

Mortality

To assess the general health of the genetically modified *Drosophila* lines, a survival assay was performed. Twenty second instar larvae were collected in a petri dish and placed at 25°C. After four days the number of pupae in each dish was counted. Between 5 and 14 petri dishes were tested for each cross, adding up to a total of 27 dishes.

Generation cgGAL4 line

Due to the frail health and resistance of the genetically modified *Drosophila* lines, an attempt was made to backcross cgGAL4 flies, in which GAL4 regulated expression is restricted to the hemocytes and fat body, with a natural population from Gotheron (44°58′N, 4°55′E, France). Flies from Gotheron (GOTH) were found to be extremely resistant in previous studies (Gerritsma et al. 2013). The general health and resistance of the cgGAL4 line was thus expected to increase in this background. cgGAL4 flies with the subsequent genotype, $+_{cgGAL4}$/y ; cgGAL4:UAS-RFP/cyo ; $+_{cgGAL4}$/TM6B were crossed with GOTH males. Non curly, tubby male offspring of this cross have then the genotype $+_{GOTH}$/y ; cgGAL4:UAS RFP/$+_{GOTH}$; $+_{GOTH}$/TM6B. Backcrossing to GOTH wildtype males once more leads to a female F2 of $+_{GOTH}$/$+_{GOTH}$; cgGAL4:UAS-RFP/$+_{GOTH}$; $+_{GOTH}$/$+_{GOTH}$, which is a fly with a GAL4 element implemented in a GOTH background. These females were crossed with flies with the double UAS-*Tep1* construct, yielding flies with the genotypes $+_{GOTH}$/y ; cgGAL4:UAS-RFP/UAS-*Tep1* ; $+_{GOTH}$/ UAS-*Tep1* (males) or $+_{GOTH}$/$+_{w1118}$; cgGAL4:UAS-RFP/UAS-*Tep1* ; $+_{GOTH}$/ UAS-*Tep1* (females). A control cross for the GAL4 driver was done by crossing the generated line with w_{1118} flies, leading to offspring with a $+_{GOTH}$/$+_{w1118}$; cgGAL4:UAS-RFP/$+_{w1118}$; $+_{GOTH}$/$+_{w1118}$ genotype. As a control for the UAS-*Tep1* flies this line was crossed with the original GOTH flies. The resistance against parasitoids, as well as the expression of both *Tep1* and *Tep2*, were tested for these lines.

Statistics

The data were analyzed with linear mixed models using the 'arm' package in R (Gelman and Su 2013; R Core Team 2014). To adhere to model assumptions, the N_0 of the genes of interest was $\log_{10}$ transformed to follow a normal distribution and subsequently normalized by subtracting the mean and dividing by the standard deviation to enable between-model comparisons. This normalized initial concentration was the response variable in the models, while the different crosses were incorporated as fixed effects. The respective qPCR plate was included as a random effect. Gene expression of sample i is defined as:

$y_i = \beta_0 + \beta_1 * Cross_1 + \beta_2 * Cross_2 + \beta_3 * Cross_3 + e_{0i}$

in which β_0 is the grand mean value of average level of expression, $\beta_{1\text{-}3}$ the slopes of respectively $Cross_{1\text{-}3}$ and e_{0ij} the residual error.

The parasitoid resistance was assessed by the melanization success. Being either successful or unsuccessful, this variable is a binomial response. The different crosses were implemented as fixed effects and the experimental date as random effect. Melanization success of sample i is defined as:

$y_i = \beta_0 + \beta_1 * Cross_1 + \beta_2 * Cross_2 + \beta_3 * Cross_3 + e_{0i}$

in which β_0 is the grand mean value of average level of expression, ind_{0j} the differences in mean responses between crosses, $\beta_{1\text{-}3}$ the slopes of respectively $Cross_{1\text{-}3}$ and e_{0ij} the residual error.

Further comparisons between different experimental conditions was done by adding these as fixed and random effects in the model. A variance-partitioning approach then aided in decomposing the total phenotypic variation in different components. The significant environmental factors humidity, air pressure and outside temperature in a nearby airport (Eelde) were added to all subsequent models using *L. victoriae*. The differences in mortality between the crosses were assessed with an ANOVA.

Results

Resistance against *Asobara tabida*

To assess the importance of *Tep1* in parasitoid resistance, we compared larvae in which *Tep1* was knocked down to two control crosses, using *A. tabida* parasitoids.

The *Tep1* expression was not significantly decreased in the knock-down cross compared to the control crosses (lm, df=2, F=3.43, p=0.115) (Figure 1). The observed variation in the knock-down cross (DaGAL4/UAS-*Tep1*) is substantial compared to the control lines, with a standard deviation of 1.164 instead of 0.188 and 0.413 respectively of the control crosses.

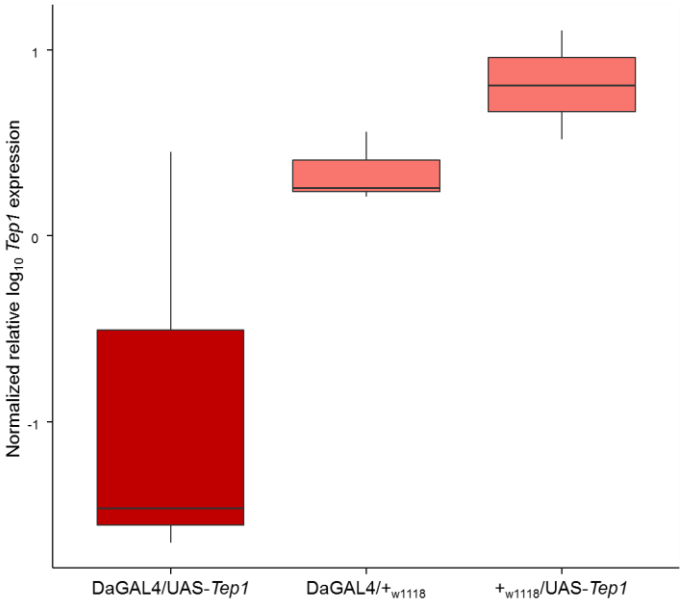

Figure 1 Log-transformed normalized *Tep1* expression of *A. tabida* parasitized larvae, at 20˚C and 24 hours post-parasitization. No significant differences were found between the different crosses (lm, df=2, F=3.43, p=0.115). The knock-down cross is indicated with a darker color.

Out of the 96 parasitized larvae, only a small proportion (0-15%) of the larvae were capable of melanizing the parasitoid egg. No statistical support for differences in melanization among the knock-down and the two control crosses was found (glmm, χ^2(df=2)=5.69, p=0.060)(Appendix Figure A1). The level of resistance was extremely low, which complicates the detection of the expected decrease in resistance in the knock-down cross.

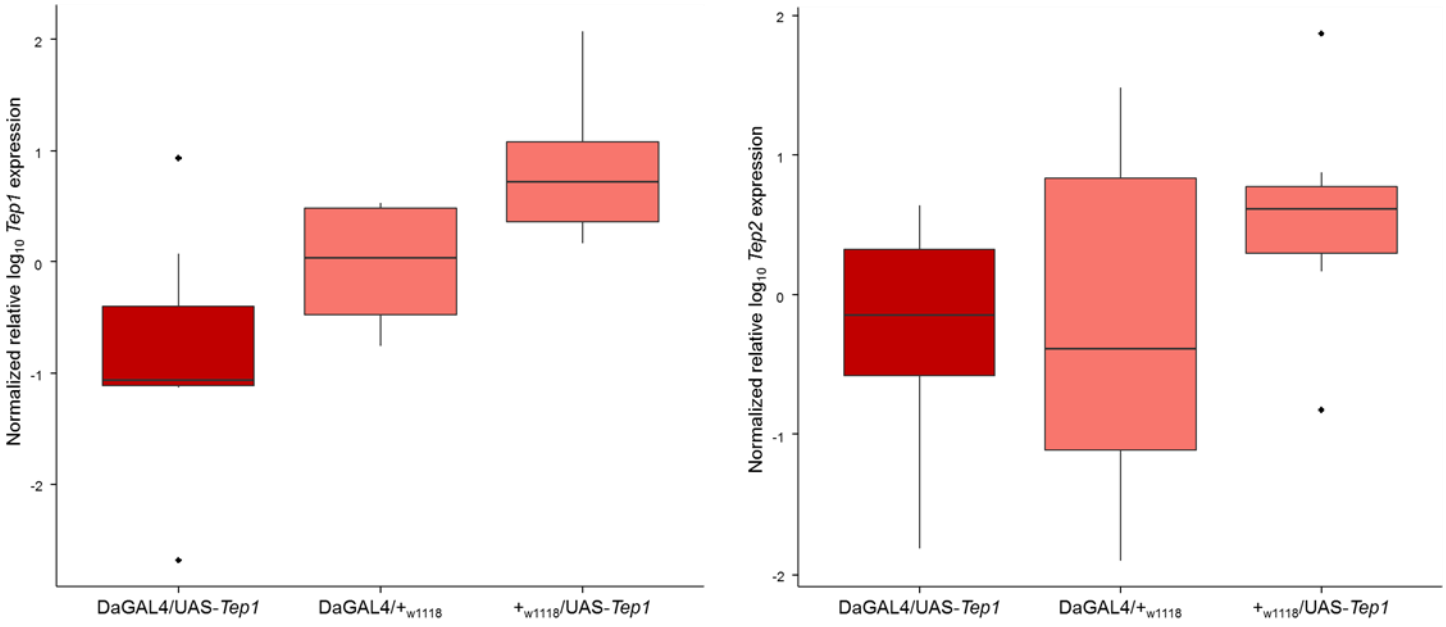

Figure 2 Log-transformed normalized gene expression of by *L. victoriae* parasitized larvae, at 25˚C and 24 hours post-parasitization. Darker shades indicate the knock-down cross. **A)** Expression of *Tep1*. DaGAL4/UAS-*Tep1* had a decreased expression compared to DaGAL4/+ (lmer, χ2(df=1)= 7.50, p=0.006) and +/UAS-*Tep1* (lmer, χ2(df=1)= 18.71, p<0.001). **B)** Expression of *Tep2.* No differences were found in Tep2 expression among the three crosses (lmer, χ2(df=2)= 2.94, p=0.230) .

Resistance against *Leptopilina victoriae*

To validate to what extend environmental variables influence the variation in melanization success,

various experimental and environmental conditions were added in the model. We incorporated experimental conditions as food quality, wasp species and temperature, as well as environmental variables as air pressure, humidity and temperature in a nearby airport (Eelde 53°8′N, 6°35′E, Groningen). This resulted in some differences among the crosses in melanization success. Only the DaGAL4/UAS-*Tep1* and DaGAL4/+ crosses and the temperature at which the experiment was conducted were significant fixed effects (respectively χ^2(df=2)= 6.64, p=0.040 and χ^2(df=1)= 4.47, p= 0.035). The air pressure, humidity, temperature (all in Eelde) as well as the date explained a significant amount of variation in melanization success and were therefore included in all subsequent models (Table 1). The residual, or within-individual, variation is zero, which can be explained by the fact that all individuals were measured once. Continuation of the experiments was done using the parasitoid species *L. victoriae*, to conduct our experiments at a more optimal temperature for

Table 1 Univariate mixed model results with estimates for the different slopes (for fixed effects) and variances (for random effects). Credible intervals are indicated in parentheses. Significant values are highlighted in bold.

	Melanization Success
Fixed effects	β (95% CI)
Intercept	-7.087 (-14.551- -0.160)
Cross (DaGAL4/+)	**0.837 (0.152 – 1.477)**
Cross (+/UAS-*Tep1*)	0.411 (-0.263 – 1.110)
Food (rich)	0.329 (-2.540 – 3.067)
Wasp species (*L. victoriae*)	-1.511 (-3.792 – 0.828)
Temperature	**0.282 (-0.001 – 0.589)**
Random effects	σ^2 (95% CI)
Date	**0.077 (0.024- 0.167)**
Air pressure (Eelde)	**0.233 (0.073 -0.485)**
Humidity (Eelde)	**5.969 (2.129-11.431)**
Temperature (Eelde)	**0.131 (0.041 -0.271)**
Residual	0.000 (0.000 – 0.000)

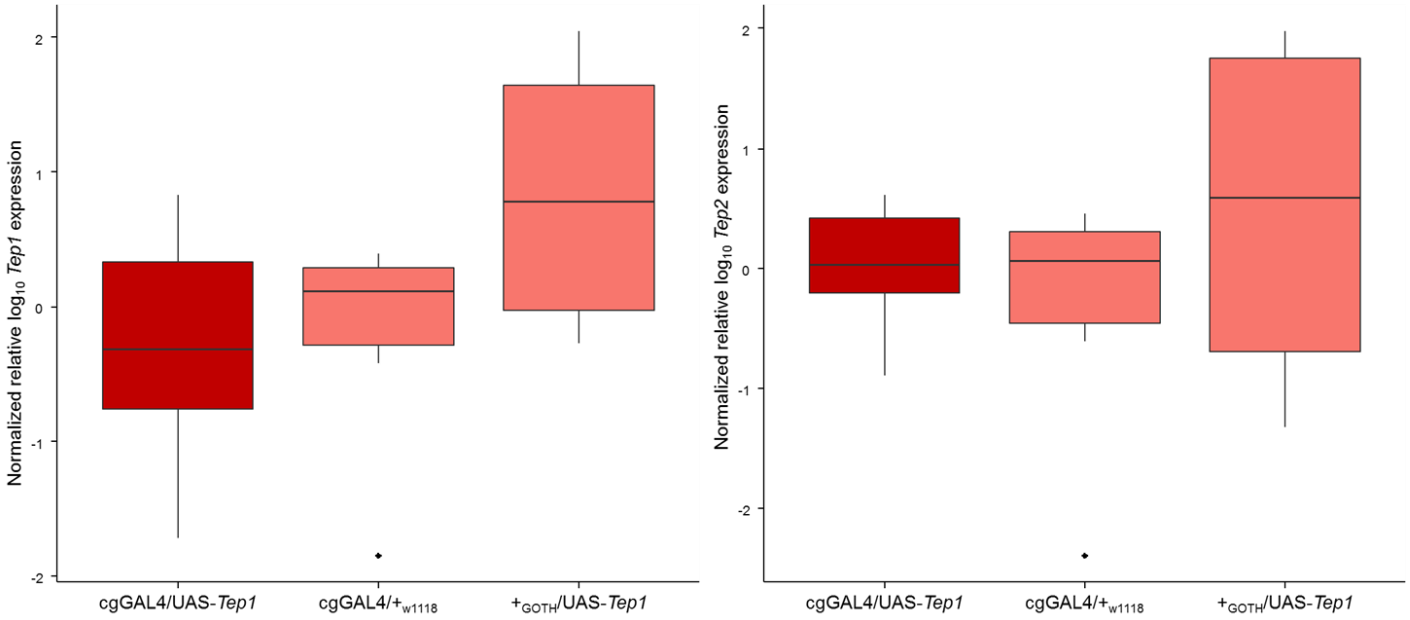

Figure 3 Log-transformed normalized gene expression of by *L. victoriae* parasitized larvae from the newly created cgGAL4 line, at 25˚C and 24 hours post-parasitization. Darker shades indicate the knock-down cross. **A)** Expression of *Tep1*. No differences were found in Tep1 expression among the three crosses (lmer, χ2(df=2)= 4.08, p=0.130). **B)** Expression of *Tep2.* No differences were found in Tep2 expression among the three crosses (lmer, χ2(df=2)= 1.72, p=0.420).

the GAL4-UAS system and to see whether the resistance of our crosses against this parasitoid would be higher. The *Tep1* expression level was significantly lower in the knockdown cross than in the two control crosses (lmer, χ^2(df=2)= 18.74, p<0.001) (Figure 2A). The knock-down DaGAL4/UAS-*Tep1* had significantly lower Tep1 expression than both DaGAL4/+ (lmer, χ^2(df=1)= 7.50, p=0.006) and +/UAS-*Tep1* (lmer, χ^2(df=1)= 18.71, p<0.001). The UAS-*Tep1* control cross showed a higher *Tep1* expression than the DaGAL4 control (lmer, χ^2(df=1)= 9.60, p=0.002), suggesting no "leaky expression" of the UAS-Tep1 construct in the absence of the GAL4 driver. The expression of Tep2 was not significantly different among the knockdown and the two control crosses (lmer, χ^2(df=2)= 2.94, p=0.230) (Figure 2B). Including the *Tep2* expression in the *Tep1* expression model, however, revealed this variable to have a significant positive slope in the model (lmer, df=1, F=28.07, p<0.001). This suggests the expression of both genes to be correlated, with larvae having an increased *Tep1* expression also having an increased *Tep2* expression. The interaction between *Tep1* and *Tep2* expression is further supported by the strong correlation between the expression of these genes (Spearman's rank correlation coefficient, ρ=0.668, p<0.001) (Appendix Figure A2).

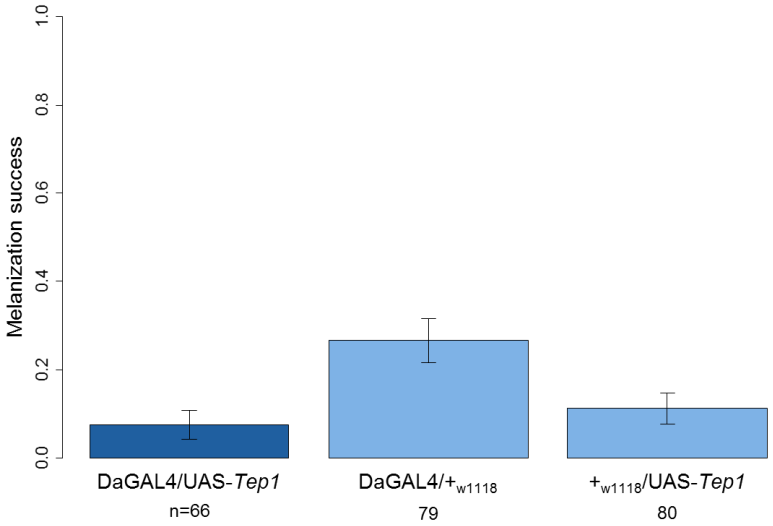

Figure 4 Melanization success of the three crosses, parasitized by *L. victoriae*. The DaGAL4/+ had a higher resistance than the DaGAL4/UAS-*Tep1* (glmm, χ2(df=1)= 9.01, p=0.003) and the +/UAS-*Tep1* (glmm, χ2(df=1)= 0.05, p=0.829).

The melanization success of all lines in this pilot experiment suggested a higher resistance against this species of parasitoids (Appendix Figure A3). Subsequent trials, however, failed to uphold this consistently (Figure 3). Nonetheless, differences were found between the three crosses (glmm, χ^2(df=2)=12.41, p=0.002). The knock-down cross DaGAL4/UAS-*Tep1* showed a significant decrease in melanization success compared to the DaGAL4/+ control cross (glmm, χ^2(df=1)= 9.01, p=0.003), but not compared to the +/UAS-Tep1 control cross (glmm, χ^2(df=1)= 0.05, p=0.829). The melanization success of the two control crosses also differed significantly from each other (glmm, χ^2(df=1)= 9.08, p=0.003). The levels of expression do not coincide with the observed levels of melanization success. Both crosses containing the UAS-*Tep1* constructs had a lower resistance, which could have indicated difficulties with these constructs. Two explanations were proposed, one that the construct showed 'leaky' expression, which would lead to a decreased *Tep1* expression in this control cross as well. The other explanation might be that the construct itself is slightly toxic, leading to a decrease in general health and resistance in the larvae. Using the qPCR data, no evidence for leaky expression of the UAS-

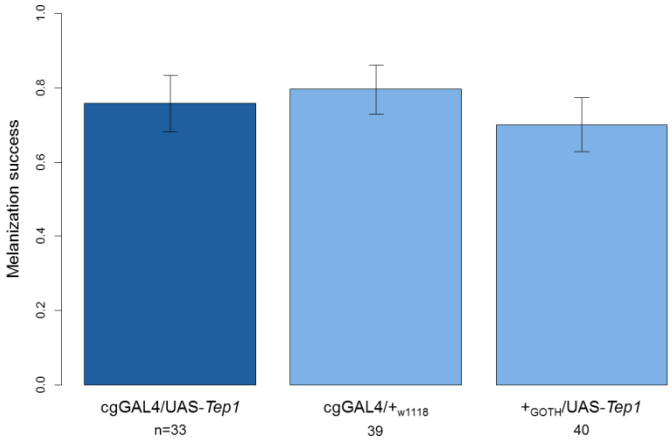

Figure 5 Melanization success of the three crosses, parasitized by *L. victoriae*. The resistance against this parasitoid was equal between all crosses (glmm, χ2(df=2)= 0.960, p=0.620)

Tep1 construct in absence of the GAL4 driver was found. The toxicity of the constructs was tested using survival assays.

No statistical support was found for differences in survival between the crosses (Kruskal-Wallis, df=2, F=0.57, p=0.573). The mortality was high for all three crosses. A trend, however, could be seen towards the DaGAL4/+ cross having a higher mortality (Appendix Figure A4).

cgGAL4 lines

To generate more resistant lines, we crossed the cgGAL4 driver in a Gotheron background. To knock the *Tep1* gene down, the generated cgGAL4 line was crossed with the UAS-*Tep1* line with the double constructs. The control crosses consisted of crossing the cgGAL4 line with the w_{1118} line and the UAS-*Tep1* line with the Gotheron flies. The resistance of these lines was tested against *L. victoriae* parasitoids.

Expression of *Tep1* did not show significant differences among the three crosses (lmer, χ^2(df=2)= 4.08, p=0.130), though a trend could be seen toward the knock-down cross DaGAL4/UAS-*Tep1* having a lower expression (Figure 3A). The *Tep2* gene showed equal expression across the different crosses as well (lmer, χ^2(df=2)= 1.72, p=0.420) (Figure 4B). The correlation between expression of both the *Tep* genes did still exist (Spearman's rank correlation coefficient, ρ=0.816, p<0.001)(Appendix Figure A5). All three crosses were found to be indeed more resistant, with melanization rates up to 80% (Figure 5). The resistance, however, was not significantly different among the knockdown cross and the two control crosses (glmm, χ^2(df=2)= 0.96, p=0.620). In this experiment, fifty larvae of a specific cross were collected in separate petri dishes (4-7 dishes per cross). Each of these petri dishes could be assessed with regard to the *Tep1*

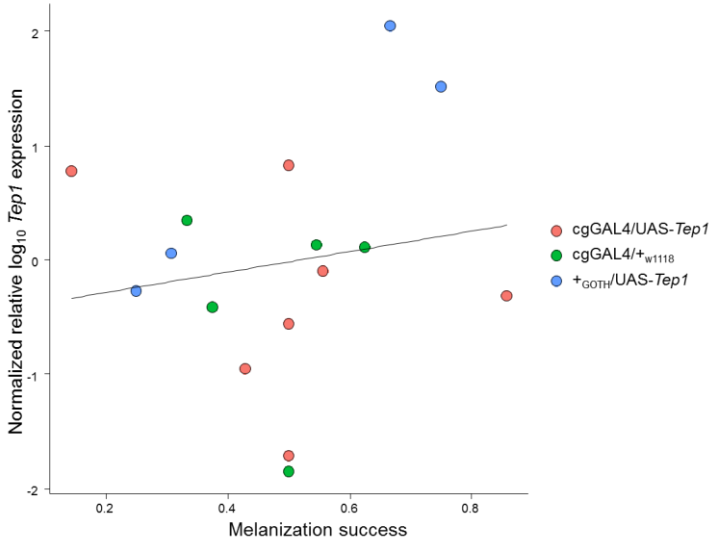

Figure 6 Correlation between the log-transformed normalized expression of *Tep1* and the melanization success in the petri dishes (Spearman's rank correlation coefficient, ρ=0.176, R^2=0.023 p=0.5137).

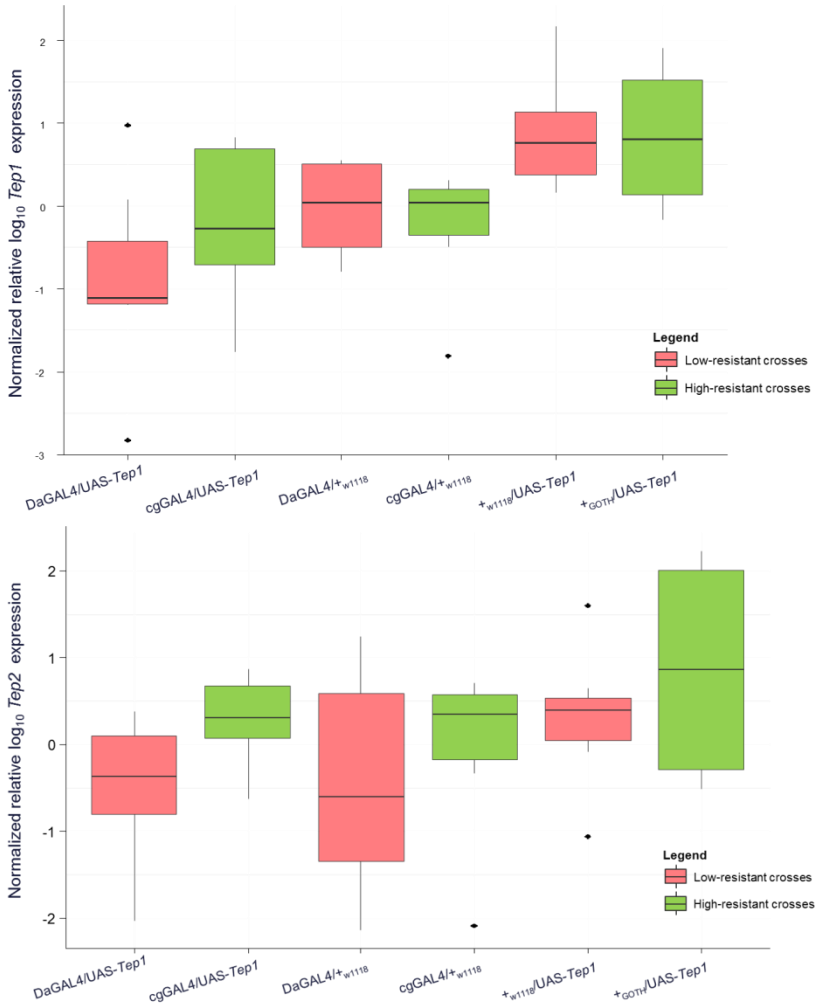

Figure 7 Comparison of log-transformed normalized expression of by *L. victoriae* parasitized larvae from the low- and high-resistant crosses, at 25˚C and 24 hours post-parasitization. **A)** Expression of *Tep1*. Differences were found between the different crosses (lmer, χ2(df=5)= 18.370, p=0.003), but no differences were found between the matched crosses of both experiments. **B)** Expression of *Tep2.* No differences were found between the crosses (lmer, χ2(df=5)= 8.050, p=0.150).

expression as well as the melanization success. As the variation of both melanization success and *Tep1* expression was high between different petri dishes, a correlation between these traits at level of the petri dishes might yet exist. This correlation between the two traits, however, was found to be weak and non-significant. This indicated the expression of Tep1 not to have any influence on the parasitoid resistance in a particular petri dish (Spearman's rank correlation coefficient, ρ=0.176, p=0.514) (Figure 6). A comparison was made between *Tep1* expression levels of the low- and high-resistant genetic backgrounds. No significant differences were found between the two matching crosses from both experiments (Figure 7A). The knock-down cross with the DaGAL4 construct, however, showed a trend towards a decrease in *Tep1* expression compared to the same cross using the cgGAL4 construct (lmer, χ^2(df=1)= 1.67, p=0.200). The expression of *Tep2* was not significantly different for the high- and low-resistant backgrounds, although there was a trend for higher *Tep2* expression in the high-resistant background (Figure 7B). In all compared crosses, similar trends of the high-resistant lines having a higher *Tep2* expression were found in all compared crosses (lmer, χ^2(df=5)= 8.05, p=0.150).

Discussion

To elucidate the functional role of the *Tep1* gene in the encapsulation response of *Drosophila melanogaster*, a knock-down of this gene was attempted using the GAL4-UAS system. No solid evidence was found for *Tep1* having a significant role in the process of encapsulation. Larvae parasitized by *A. tabida* were rarely able to melanize the parasitoid egg. As the knock-down of *Tep1* was expected to lead to a decreased resistance, the inability of the control crosses to encapsulate *A. tabida* parasitoid eggs thwarted the drawing of any solid conclusions. Parasitization by *L. victoriae* did yield a significant decrease in Tep1 expression in the knock-down cross compared to the control crosses, but this could not be linked to differences in parasitoid resistance. Though the assessed resistance against parasitoids was lower in the crosses with the UAS-*Tep1* constructs, there was no evidence of leaky expression or toxicity of the constructs. The crosses using a cgGAL4 construct in a Gotheron background showed a remarkable increase in melanization success. This success was not reflected by any differences in *Tep1* expression, but might be attributed to differences in genetic background. As in none of the experiments differences in *Tep2* expression between the knock-down and control crosses were found, compensation by this gene of the potentially redundant *Tep1* was improbable.

Tep1 knock-down

The knock-down of the *Tep1* gene in larvae parasitized by *A. tabida* showed a trend towards being decreased with respect to the control crosses. This trend, however, was not significant and resembled the results of Salazar-Jaramillo et al. (in preparation). Due to the low sample size, however, we decided that this reduction was adequate for continuation of the experiment. The knock-down, however, was expected to be more efficient with the inclusion of a second UAS-*Tep1* construct compared to the single construct used in the experiments done by Salazar-Jaramillo et al. (in preparation). Consequently, the question remains what the additive effect of including a second UAS-*Tep1* construct in our genetically modified lines is. Even though the GAL4-UAS constructs are temperature dependent, a significant knock-down was expected at 20°C in the double construct lines.

Further experiments did show a successful knock-down of *Tep1*, though the observed variation remained high, due to a wide, lognormally distributed, spread of expression values. Using the cgGAL4 in the knock-down was not successful in terms of decreasing the expression of the target gene. Important, though, is to recognize that cgGAL4 is only expressed in the fat body and the hemocytes of the *Drosophila* larvae and that the knock-down is expected to only happen in these tissues (Asha et al. 2003). While *Tep1* is mostly expressed in the fat body, substantial expression is also found in the midgut, tubules and trachea (Chintapalli et al. 2007). As RNA extractions were done on whole larvae, this could be a cause of overestimation of the actual expression in the targeted tissues. Furthermore, the assumption that the *Tep1* gene influences the encapsulation and/or melanization of parasitoid eggs and should therefore be mostly expressed in the larval hemocytes might be inaccurate, as the exact function of this gene is not known yet. The knock-down induced by the cgGAL4 construct might thus not be in the

appropriate tissues and therefore have no effect on the melanization response. Ideally, future experiments should therefore validate the main function of *Tep1* to be in the hemocytes and thus the use of the cgGAL4 driver in *Tep1* research.

Several factors could have led to suboptimal activation of the GAL4-UAS system. Although its efficiency has been found to be variable, the GAL4-UAS system is widely used in gene expression research (Traven et al. 2006). In the yeast *S. cerevisiae*, GAL4 is a regulatory transcriptional activator related to galactose transport in cells (Traven et al. 2006). The upstream activating sequences (UAS) through which this activator operates vary in their relative affinity for this activator and thus in their expression (Lohr et al. 1995). These differences in affinity are not yet well explained. Proposed factors are the chromatin structure of the target site or the vicinity and number of other binding sites (Lohr et al. 1995; Traven et al. 2006). Furthermore, the phosphorylation of GAL4 has also been found to influence activational power, with high phosphorylation leading to an increased expression of the target gene. As the interaction between GAL4 and UAS is intrinsically limited in strength, cellular conditions might also have an significant importance. Presence of glucose, for instance, has been found to repress GAL4 expression, while glycerol has an enhancing effect on the expression of this regulator (Lohr et al. 1995).

Suggestions that one GAL4 driver would lack the capability of providing sufficient product to bind multiple UAS binding sites have not been confirmed in previous research. Contrary, an increased number of binding site was found to boost the transcript levels (Pfeiffer et al. 2010). Two adjacently placed UAS sites would even synergistically increase their total activity (Giniger and Ptashne 1988).

Strong correlation Tep1 and Tep2

The strong, positive correlation between expression of *Tep1* and *Tep2* was an interesting result. The expression of *Tep2* itself did not vary between crosses or experiments, indicating that Tep1 knockdown did not result in off-target knockdown of Tep2. The data are in agreement with research showing that both genes are upregulated in case of immune challenge (Schlenke et al. 2007). However, there was no indication for a possible redundancy of the *Tep1* gene and compensation by *Tep2*. Even though gene redundancy is fairly widespread, from an evolutionary point of view it might be seen as a paradox. Since true redundancy would be expected to lead to an accumulation of deleterious mutations, other mechanisms must intervene to attain an evolutionary stable state (Nowak et al. 1997). Several plausible and not mutually exclusive scenarios have been proposed. Certain is though, that a delicate balance between the mutation rates and efficacy of all genes involved is to be maintained. Combined with pleiotropy or as a back-up system of developmental errors, redundant genes are most likely to be preserved (Nowak et al. 1997). Even though *Tep2* expression gave no indication, further micro-array analysis or RNA sequencing could be used to determine the potential redundancy of *Tep1*. Comparing the transcriptome of both *Tep1* knock-down and control flies, significantly upregulated genes in the former would be candidates for genes potentially compensating for the silenced *Tep1* gene. Parasitoid

resistance assessments with double or triple knockdowns could then be attempted to confirm the role of *Tep1* in the encapsulation process.

Experimental conditions

Remarkable is how little the outcome of the experiments was influenced by the different experimental conditions we used, such as host diet, temperature and wasp species. The larvae were hypothesized to be feeble and highly sensitive to environmental change and thus predicted to be responsive to changes in external conditions. Various factors have been previously linked to influencing the process of encapsulation in insects, including host diet (e.g. host plant species), temperature and symbionts (Blumberg and Van Driesche 2001; Fytrou et al. 2006; Karimzadeh and Wright 2008). The observation of an immense day-to-day variation in our experiments suggested an importance of experimental and external factors. None of this variation appeared to be explained by the wasp species used and the host diet. It has to be noted, however, that only two types of food were tested. These types differed mainly in their protein content. Besides, apart from the food source, larvae were also provided with yeast paste. Consuming mostly the more liquid paste until the third instar stage, little of the provided food might have been consumed.

The absence of significant influence of wasp species on encapsulation is striking. As the immune response has both a parasitoid-specific and a generic component, resistance against one parasitoid species usually does not imply resistance against another (Dubuffet et al. 2009). Resistance of the host and virulence of the wasps are therefore normally seen as relative terms, as they depend on the interaction between the genotypes of both (Dubuffet et al. 2007).

Our results lead to the conclusion that the study system by itself might be relatively robust. Implications are that the comparability between different experiments with slight differences in experimental setup might be higher than assumed in previous studies (Prévost 2009).

Low vs. high resistant lines

As evidence linking *Tep1* expression to parasitoid resistance could not be found using the knock-down crosses, another potential method to shed light on the underlying mechanisms of the encapsulation response would be to compare expression between the low- and high-resistant lines. Surprisingly, comparisons between these lines did not yield differences in *Tep1* expression, while *Tep2* levels showed a trend to be higher in high-resistant lines. As *Tep2* is essential for phagocytosis of *E. coli* bacteria, this trend might be due to an improved general health of these lines. The general health of the host has been associated with the outcome of host-parasite interactions before (Ayres et al. 2008). Thus while *Tep1* might not play a noticeable role in the encapsulation response, the introduction of a Gotheron genetic background might lead to an improved health and thus higher resistance.

Conclusions and future studies

Future research should validate the ability of the GAL4-UAS system to uncover the role of *Tep1* in parasitoid resistance. Whether the use of this system is difficult due to a limited influence of *Tep1* on

the encapsulation of parasitoid eggs, excellent efficiency of even small amounts of Tep1 protein or potential redundancy of the gene, remains to be revealed. A knock-out experiment combined with microarray analyses might shed light on the potential redundancy. Alternatively, as logical continuation of RNAi experiments in high-resistant lines, *Tep1* expression might be increased in low-resistant lines to assess the effects this has on the parasitoid resistance. The ultimate disclosure of the role of *Tep1* will elucidate the pathways in which the *Drosophila* immune response acts. Furthermore, this might resolve the evolutionary pathways of the highly variable immune receptor genes in general as well as specifically on the co-evolutionary dynamics of *Drosophila* and its parasitoids. Further research will be necessary to validate and extend the results of this study and to assess a range of parasitoid resistance related genes into more depth.

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Appendix A - Figures

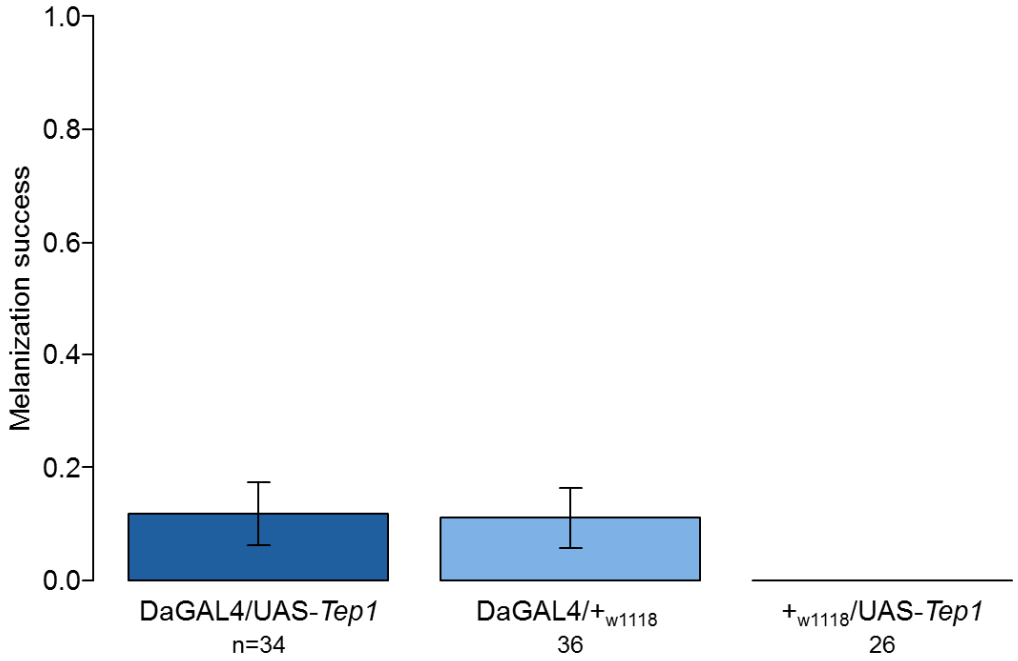

Figure A1 Melanization success of the three crosses, parasitized by *A. tabida*. The knock-down cross is indicated with a darker color. No significant differences could be found between the crosses (glmm, $\chi 2(df=2)=5.69$, $p=0.060$).

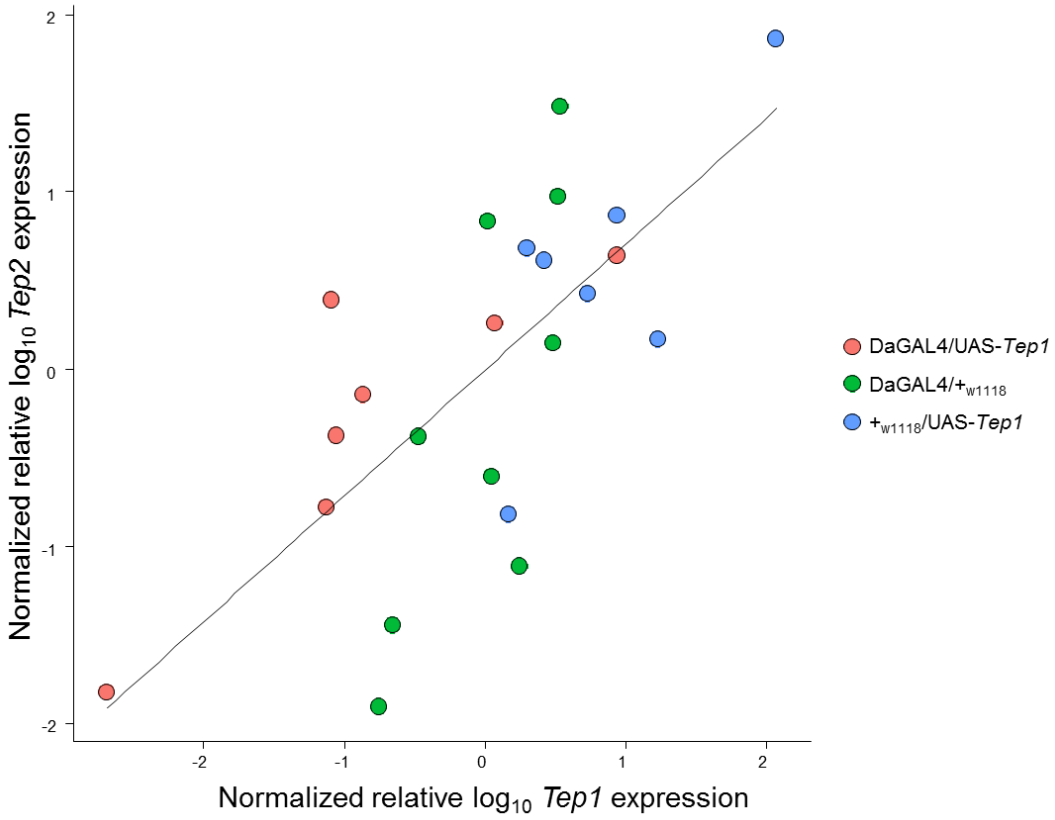

Figure A2 Correlation between the log-transformed normalized expression of *Tep1* and T*ep2* of the knock-down and control crosses using the DaGAL4 driver (Spearman's rank correlation coefficient, $\rho=0.668$, $R^2=0.482$, $p=0.0007$).

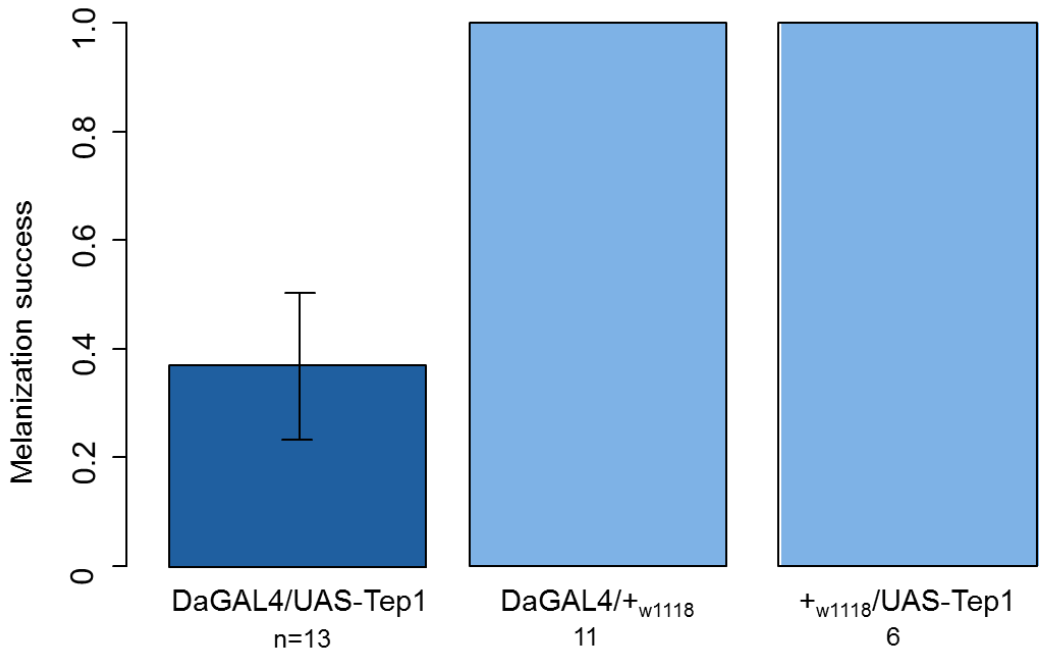

Figure A3 Melanization success of the three crosses in the pilot experiment, parasitized by *L. victoriae.* The knock-down cross is indicated with a darker color.

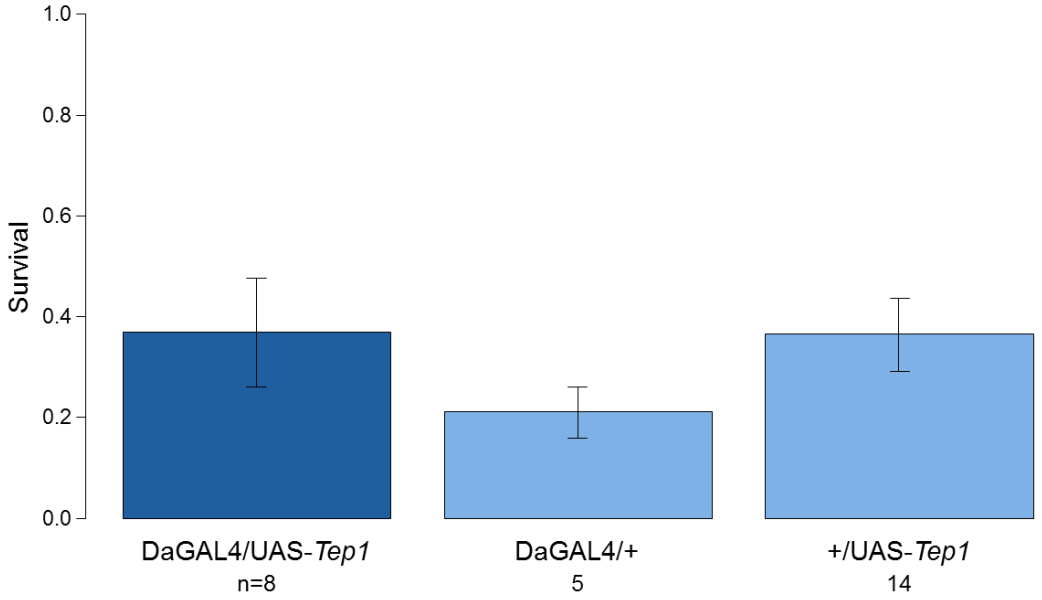

Figure A4 Survival of unparasitized larvae of the three crosses in the survival assay. The knock-down cross is indicated with a darker color. No significant differences were found among the crosses (Kruskal-Wallis, df=2, F=0.57, p=0.573).

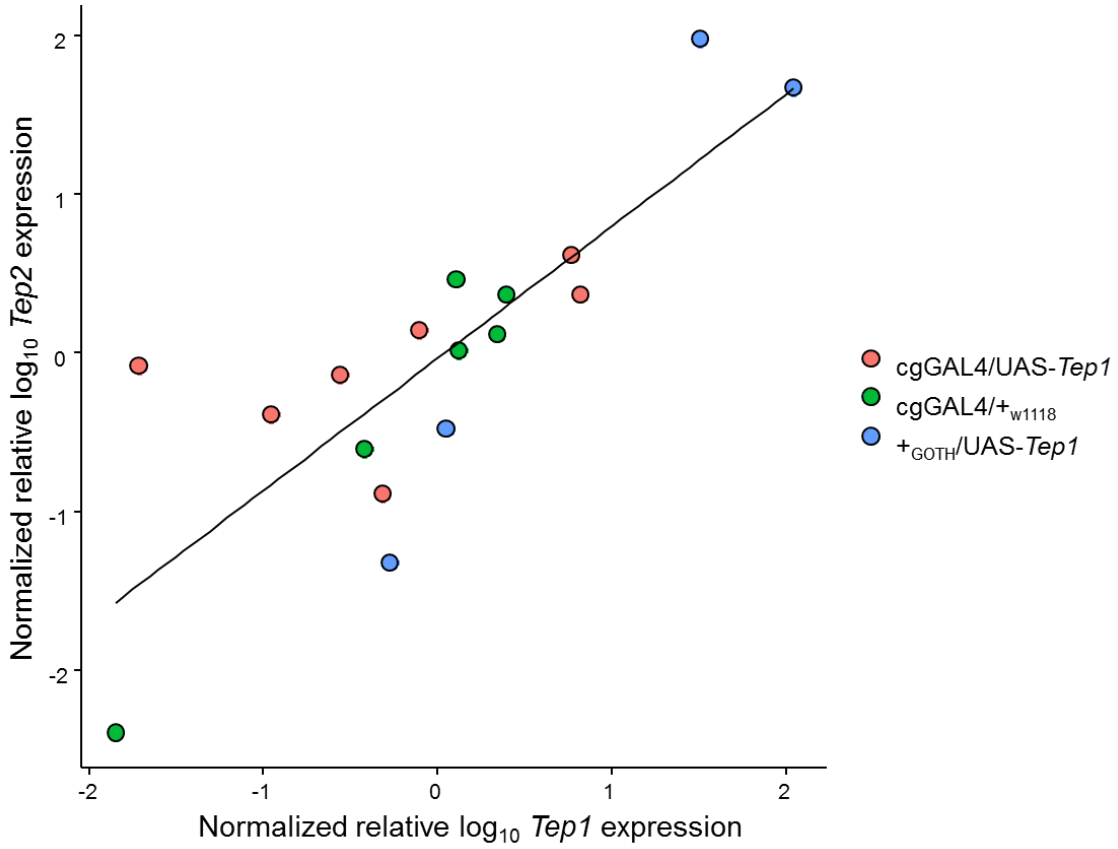

Figure A5 Correlation between the log-transformed normalized expression of *Tep1* and T*ep2* of the knock-down and control crosses using the cgGAL4 driver (Spearman's rank correlation coefficient, ρ=0.816, R^2=0.643, p<0.001).