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Endogenous egg immune defenses in the yellow mealworm beetle (*Tenebrio molitor*)

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ABSTRACT

In order to survive microbe encounters, insects rely on both physical barriers as well as local and systemic immune responses. Most research focusses on adult or larval defenses however, whereas insect eggs are also in need of protection. Lately, the defense of eggs against microbes has received an increasing amount of attention, be it through endogenous egg defenses, *trans*-generational immune priming (TGIP) or parental investment. Here we studied the endogenous immune response in eggs and adults of *Tenebrio molitor*. We show that many immune genes are induced in both adults and eggs. Furthermore, we show that eggs reach comparable levels of immune gene expression as adults. These findings show that the eggs of *Tenebrio* are capable of an impressive endogenous immune response, and indicate that such inducible egg defenses are likely common in insects.

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1. Introduction

To combat infection, insects rely on both physical barriers as well as local and systemic immune responses. Much research on immune responses has focused on adult and larval insects. However, for eggs to survive, they too need to be protected against natural enemies, such as parasites, predators and microbes. Well known mechanisms of defense are the mother's choice of oviposition site (Janz, 2002), physical barriers covering the eggs such as the egg shell (Stanley and Miller, 2006), and parental investment in the form of defensive secretions applied to the eggs (Boos et al., 2014; Hilker and Meiners, 2002). Recent advances however, have made it increasingly clear that insect eggs themselves are far from defenseless and can harbor strong endogenous defenses (Gorman et al., 2004; Jacobs et al., 2014a; Jacobs and Van Der Zee, 2013).

One form of endogenous defense is the secretion of the serosal cuticle by the extraembryonic serosa, which protects against dehydration (Jacobs et al., 2013; Machida and Ando, 1998). However, due to the high expression of the NF-kappa-B factor *dorsal*, which is well known for its role in immune gene induction (Ferrandon et al., 2007), Chen et al. proposed that the serosa might also protect against infection (Chen et al., 2000). Indeed, immune gene expression was shown to be restricted to the extraembryonic regions of the egg in *Manduca sexta* (Gorman et al., 2004). By using RNAi to prevent the development of the serosa (van der Zee et al., 2005), we were able to show that the endogenous innate immune response of the *Tribolium castaneum* egg is completely dependent on the extraembryonic serosa (Jacobs et al., 2014a; Jacobs and Van Der Zee, 2013). Immune gene activity in insect eggs is not only activated by a pathogen attack, but it can also be enhanced by parental immune priming. This transgenerational immune priming (TGIP) increases immune activity in eggs laid by immune challenged parents, even in the absence of egg infection (Little and Kraaijeveld, 2004). Priming prepares the eggs for the impending danger by triggering differential expression of immune-related genes in eggs and/or via the direct transmission of immune factors into eggs (Freitak et al., 2014, 2009; Knorr et al., 2015; Trauer-Kizilelma and Hilker, 2015; Trauer and Hilker, 2013; Zanchi et al., 2011).

To investigate whether TGIP and endogenous protection can be complementary strategies for egg defense, we studied immune gene expression in the eggs of the yellow mealworm beetle (*Tenebrio molitor*). This species is well known as a model for innate immunity (Chae et al., 2012; Dobson et al., 2012; Goltsev et al., 2009; Johnston et al., 2014; Lee et al., 1996; Moon et al., 1994; Park et al., 2007, 2006; Yu et al., 2010; Zhang et al., 2003; Zhu et al., 2013, 2014), but has also received considerable attention with respect to TGIP (Dubuffet et al., 2015; Moreau et al., 2012; Moret, 2006; Zanchi et al., 2012). Here we show that the eggs of *T. molitor* can induce immune genes, including antimicrobial peptides (AMPs), to high levels upon bacterial challenge. We also show that these same genes are induced in adults, and that the expression in eggs reaches comparable levels to that of adults for several of the genes tested. Our results provide important insights into egg defense strategies, highlighting that parental protection in the form of TGIP does not exclude potent endogenous defenses. These findings stress the need for detailed study of egg defense mechanisms to fully appreciate the balance between parental input and endogenous defenses.

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2. Materials and methods

2.1. Insect rearing

Final-instar larvae of *Tenebrio molitor* were purchased from a commercial supplier (Live Foods UK) and maintained in an insectary at 26 ± 2 °C under a 12:12 h light:dark cycle. Larvae were provided with ad libitum access to Progrub (Livefoods Direct Ltd) with 5% w/v brewer's yeast, and supplemented with fresh potato once per week. Pupae were collected within 2–3 days of pupation before being sexed and maintained in isolation within grid box containers. Upon imaginal eclosion, adults were provided with Progrub, yeast and fresh potato. Mating groups were established using 10–14 day old adults. Groups consisted of ca. 100 individuals in a 1:1 sex ratio, maintained in sieved wholemeal flour supplemented with 5% w/v brewer's yeast.

2.2. Infection of eggs

To stimulate immune gene induction in eggs, we used a concentrated mixture of *Escherichia coli* (Gram-negative) and *Micrococcus luteus* (Gram-positive), which was created by culturing each bacteria (provided by Simon Foster, University of Sheffield, UK) to carrying capacity before pelleting, removing the supernatant, and mixing both pellets (Jacobs and Van Der Zee, 2013). In order to collect eggs, we provided mating groups with fresh flour. After 24 h, adults were removed and the flour sieved to collect eggs. Eggs were allowed to develop at 26 ± 2 °C for 72 h. At this stage the serosa is present (Ewest, 1937); this extraembryonic membrane has been shown to be crucial for the egg immune response (Jacobs et al., 2014a; Jacobs and Van Der Zee, 2013). Eggs were either pricked with a 1 μm tungsten needle (Fine Science Tools) dipped in the concentrated *E. coli*/*M. luteus* culture (undiluted), pricked with a sterile needle, or remained unchallenged. Before RNA extraction, eggs were incubated for 6 h at 26 ± 2 °C. Eggs were briefly washed in a 0.5% hypochlorite solution to sterilize the outside surface before RNA extraction. We collected 9 samples, with 3 biological replicates for each of the 3 treatments.

2.3. Infection of adults

In order to compare the immune gene induction of eggs with that of adults we also infected adult beetles. For the adults we used a different immune stimulus than in the eggs. We grew overnight colonies of *E. coli* and *M. luteus* in LB and *Serratia entomophila* (Gram-negative) in Caso medium. These overnight cultures were spun down and resuspended in PBS and diluted to a concentration of 2.000 CFU/μl. After treatment, adults were incubated for 6 h at 26 ± 2 °C before RNA extraction.

We collected samples for five different treatments: untreated individuals, PBS injected individuals, *E. coli* injected individuals, *M. luteus* injected individuals and *S. entomophila* injected individuals. We injected 5 μl of solution (10.000 CFU) into the haemocoel between the third and fourth abdominal sternites using a Hamilton syringe. Six beetles were taken randomly from a mating group (containing both males and females) for each sample. In total we collected 15 samples, with 3 biological replicates for each of the 5 treatments.

2.4. Sequences of immune-related genes and primers used for real-time quantitative RT-PCR (qRT-PCR)

In order to screen the entire repertoire of antimicrobial peptides (AMPs) of *Tenebrio molitor* we obtained the dataset of Johnston et al. (2014) and created a local blast database (utilizing their File S2). We screened this dataset for potential AMPs, pattern recognition proteins, toll receptors and gram-negative binding proteins (GNBPs). We only included those sequences of which we were certain of their identity. We identified 16 AMPs, one *GNBP*, one toll receptor and 2 pattern recognition receptors (File S1 and S2). Of these genes, attacin D resembles the known AMP tenecin 4 (accession BAL04117.1; Chae et al., 2012). However, our sequence is incomplete (File S2), and has 1 amino acid difference in the predicted mature peptide. In addition, in contrast to the sequence we have obtained from the Johnston et al. (2014) dataset, a large portion of the propeptide is missing in the published sequence. Furthermore, there are many differences in the signal peptide, indicating that our attacin D might not be the same gene as the published tenecin 4 but rather represent a closely related new gene. Defensin 1 is also known as tenecin 1 (accession Q27023.1; Moon et al., 1994). In this study we choose to adopt attacin D and defensin 1, respectively, for these genes, as these names confer information about the gene families discussed. Furthermore, we were able to identify coleoptericin A-C (accession All26031.1, All26032.1 and All26033.1; Zhu et al., 2014), attacin C (Dobson et al., 2012), tenecin 3 (accession Q27270.1; Lee et al., 1996), *PGRP-SA* (accession BAE78510.1; Park et al., 2006) and *PGRP-SC2* (accession BAJ23047.1; Yu et al., 2010). We also found attacin A and B (described as attacin A and C in Johnston et al., 2014), cecropin, defensin 2 and 3, thaumatin 1-3, a putative defense protein hdd11, a *GNBP* and a toll receptor. We used the ribosomal protein L13a (RPL13a) as a reference gene for qRT-PCR. We designed primers for qRT-PCR using the NCBI's primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer specificity was checked against the local database. The primers of the sequences used in this study can be found in Table S1.

2.5. RNA extraction and qRT-PCR

For each adult sample, 6 adult beetles were ground into a fine powder in liquid nitrogen. Total RNA of approximately 50 mg of powder from adults or approximately 45 eggs was extracted using TRIzol extraction (Invitrogen), after which the RNA was purified with the RNeasy kit (Qiagen). The quality of RNA preparation was confirmed spectrophotometrically. One microgram of total RNA was used for cDNA synthesis. DNA digestion and first strand cDNA synthesis were performed using the iScript gDNA clear synthesis kit (Biorad). Each qRT-PCR mixture (20 μl) contained 2.5 ng of cDNA, and the real-time detection and analyses were done based on SYBR green dye chemistry using the SsoAdvanced Universal SYBR Green Supermix (Biorad) and a CFX96 thermocycler (Biorad). Thermal cycling conditions used were 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s; this was followed by dissociation analysis of a ramp from 65 °C to 95 °C with a read every 0.5 °C. Relative quantification for each mRNA was done using the Livak-method (Livak and Schmittgen, 2001). The values obtained for each mRNA were normalized by RPL13a mRNA amount. Total RNA for each treatment was isolated three times (biological replication) and each sample was measured by qRT-PCR twice (technical replication).

3. Results

3.1. Induction of immune genes in eggs

To assess whether the eggs of *T. molitor* are able to induce immune genes, we measured immune gene expression after sterile in-

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jury (sterile needle) and after septic injury (*E. coli* and *M. luteus* mixture, Fig. 1). When comparing gene expression to untreated eggs, we saw a high induction of immune genes in the infected eggs of *T. molitor* (Fig. 2, Fig. S1). All AMP genes measured, except for thaumatin 1-3 and tenecin 3, are induced upon both sterile injury and septic injury (Fig. 2, Fig. S1). Several AMPs, such as the attacins A-D and coleoptericin A-C are induced more than 500 fold after septic injury (Fig. 2a,c and Figs. S1a–e). While we could not detect any induction of *GNBP*, both *PGRPs* were induced, as was the toll gene we measured (Fig. 2f–h and Fig. S1k). These results clearly demonstrate that the eggs of *T. molitor* are able to induce immune genes upon infection.

3.2. Induction of immune genes in adults

In order to verify that the same genes are immune inducible in *T. molitor* in general, we measured their expression in adults after sterile injection (PBS), infection with *E. coli*, infection with *M. luteus* and infection with *S. entomophila* (Fig. 1). In general, the expression pattern in adults resembled those found in the eggs (Fig. 3, Fig. S2). However, notable exceptions are the clear induction of the toll receptor, *PGRP-SC2* and *PGRP-SA* in eggs, which are at most moderately induced in adults. Furthermore, several AMPs show a higher induction in eggs than in adults. The expression of immune genes in adults shows that in general the Gram-negative bacteria *E. coli* and *S. entomophila* elicit a stronger response than the Gram-positive bacterium *M. luteus*.

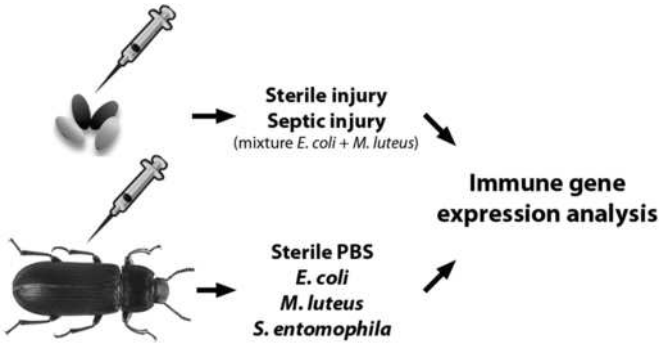

Fig. 1. Experimental setup. We collected RNA from eggs and adults of *T. molitor*. Eggs were either sterilely injured or septically injured, these samples were compared to untreated eggs. In adults we collected samples for beetles injected with sterile PBS, *E. coli*, *M. luteus*, or *S. entomophila* and we compared these to samples from untreated adults. For details see section 2. Materials and methods.

3.3. Egg induction compared to adults

Several AMPs were induced more in eggs than in adults. Because we found no differences in the expression level of the reference gene (RPL13a) between the egg and adult samples, we compared expression levels in infected adults (*E. coli* or *M. luteus*) directly to the expression in infected eggs (septic injury). We find that while for all genes measured, the expression in adults is equal to or higher than the expression in eggs, eggs reach expression levels close to those of adults in many cases (attacin A-D, coleoptericin A-C, defensin 1,3, *PGRP-SA* and *SC2*, Fig. 4 and Fig. S3). Interestingly, the genes that are not induced upon infection in adults, namely cecropin, tenecin 3 and thaumatin 1-3, are expressed much higher in infected adults than in infected eggs (Fig. 4b,e and Fig. S3 h-j). While these genes are also barely induced in eggs, it is likely that these AMP genes are constitutively expressed at high levels in adults. Despite the induction of *toll* in the eggs and the lack of induction in adults, this *toll* gene is still expressed consistently higher in adults. Overall these results show that despite larger fold-change differences in eggs, transcript levels in eggs do not surpass transcript levels in adults.

4. Discussion

In this study we investigated the endogenous immune response of both adults and eggs in *Tenebrio molitor*. We found clear induction of most immune genes tested in both eggs and adults. Thus, similar to what has been found in *Tribolium castaneum* (Jacobs et al., 2014a;

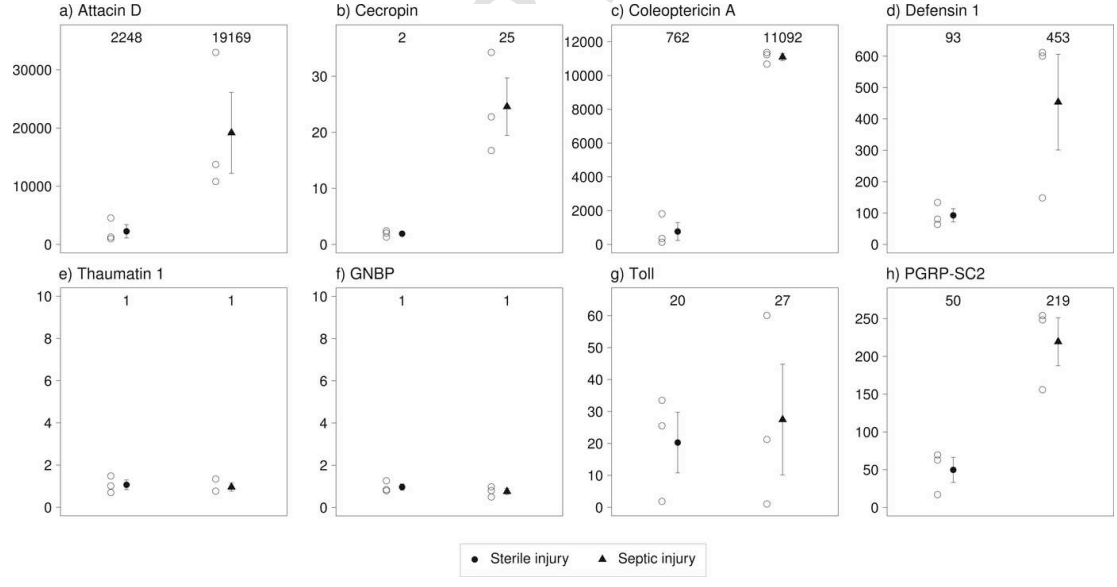

Fig. 2. Immune gene expression in eggs. Shown is the average fold-change based on two technical replicates for each biological replicate (round open circles), the average fold-change (number above the datapoints) and the average ± SEM (closed circle for sterile injury, closed triangle for septic injury). a) Attacin D, b) Cecropin, c) Coleoptericin A, d) Defensin 1, e) Thaumatin 1, f) GNBP, g) Toll, h) PGRP-SC2.

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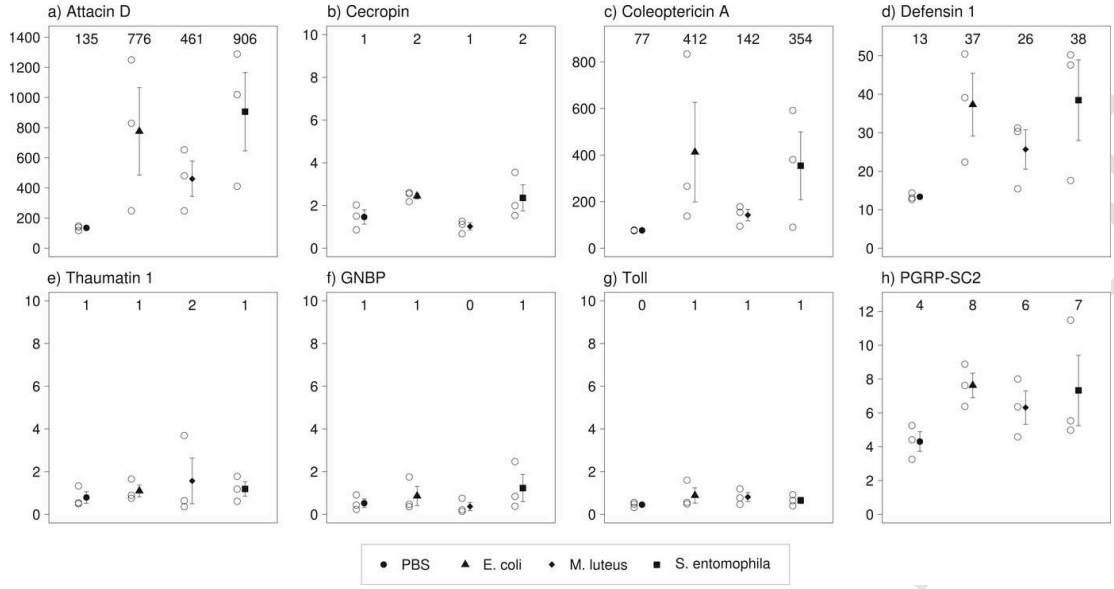

Fig. 3. Immune gene expression in adults. Shown is the average fold-change based on two technical replicates for each biological replicate (round open circles), the average fold-change (number above the datapoints) and the average ± SEM (closed circles for PBS, closed triangle for *E. coli*, closed diamond *M. luteus*, closed square *S. entomophila*). a) Attacin D, b) Cecropin, c) Coleoptericin A, d) Defensin 1, e) Thaumatin 1, f) GNBP, g) Toll, h) PGRP-SC2.

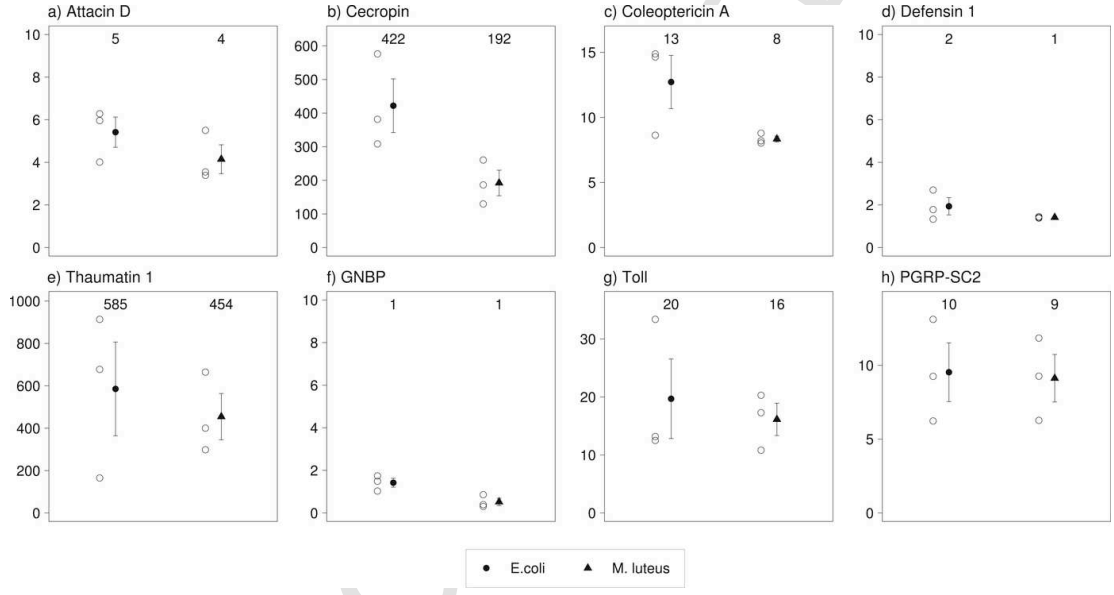

Fig. 4. Immune gene expression in adults compared to infected eggs. Shown is the average fold-change based on two technical replicates for each biological replicate (round open circles), the average fold-change (number above the datapoints) and the average ± SEM (closed circle for *E. coli*, closed triangle for *M. luteus*). a) Attacin D, b) Cecropin, c) Coleoptericin A, d) Defensin 1, e) Thaumatin 1, f) GNBP, g) Toll, h) PGRP-SC2.

Jacobs and Van Der Zee, 2013), eggs of *T. molitor* are able to induce immune genes and reach expression levels close to that of adults.

The induction of *PGRP-SA* and *PGRP-SC2* in eggs and adults is in line with their function as a pattern recognition protein and scavenger receptor, respectively (Park et al., 2006; Yu et al., 2010). *PGRP-SA* in *T. molitor* is able to recognize both Lys- and DAP-type peptidoglycans (Park et al., 2006), which are associated with Gram-positive and Gram-negative bacteria. This gene is indeed induced in adults in response to both types of challenges (*M. luteus, E. coli/S. entomophila*). *PGRP-SA* is induced in eggs to levels equal to adults in both *T. molitor* and in *T. castaneum* (Jacobs and Van Der Zee, 2013), indicating its importance. It is interesting to see that in both *T. castaneum* and *T. molitor* eggs, a toll gene is induced (Jacobs et al., 2014a). However without clear evidence of which specific *T. molitor* toll sequence is responsible for immune gene induction, no clear conclusions can be drawn. The induction of AMPs also shows comparable albeit higher levels to the published literature (Chae et al., 2012; Moon et al., 1994; Zhu et al., 2014).

Thaumatin 1-3, cecropin and tenecin 3 were not induced in adults, and only cecropin was induced moderately in eggs. Cecropins have broad-spectrum activity against Gram-negative and Gram-positive bacteria, as well as fungi (Yi et al., 2014). We also found a high baseline expression for cecropin in *Tribolium castaneum* (Jacobs and Van Der Zee, 2013), indicating that these AMPs might be important for

prevention of infection in adults. Both thaumatins and tenecin 3 are antifungal proteins (Jung et al., 1995; Lee et al., 1996; Vigers et al., 1992) and were not expected to be induced upon bacterial infection. They are however expressed at much higher levels in adults than in eggs. Flour habitats are known to contain a high diversity and abundance of bacteria, yeasts and fungi (Borjesson et al., 1989; Yezerski et al., 2005). It is likely that these beetles maintain a constant high-level defense against fungi that are naturally present in their environment.

Previously it was found that defensin 1 (called tenecin 1 in Dubuffet et al., 2015), is responsible for the *trans*-generational immune priming effect (Dubuffet et al., 2015). Due to the exclusive bactericidal effect on Gram-positive bacteria of defensin 1 (Moon et al., 1994), TGIP in *Tenebrio molitor* is only effective against Gram-positive bacteria. Even when induced with Gram-negative bacteria, extracts from primed eggs are only effective against Gram-positive bacteria (Dubuffet et al., 2015). Contrary to defensin 1, attacins are known for their bactericidal activity against Gram-negative bacteria (Yi et al., 2014) and coleoptericins for their activity against both Gram-negative and Gram-positive bacteria (Lee et al., 2013). The very high induction of attacins, coleoptericins and defensins in infected eggs indicates that, although experiments to verify it are needed, *Tenebrio* eggs are likely well protected against both Gram-negative and Gram-positive bacteria.

With the clear endogenous induction of immune genes in eggs of *Tenebrio molitor* we provide another example of an insect species where eggs are capable of mounting an immune response and defend against infection. Previously, egg immune gene induction has been found for *Manduca sexta* (Gorman et al., 2004) and this immune response also decreases the number of eggs successfully parasitized by a parasitoid wasp (Abdel-latief and Hilker, 2008). We have previously also shown that the eggs of *Tribolium castaneum* can defend against infection, and that this defense limits bacterial growth rate (Jacobs et al., 2014a; Jacobs and Van Der Zee, 2013). This was however not true for *Drosophila melanogaster*, which lacks a serosa (Jacobs and Van Der Zee, 2013). The burying beetle *Nicrophorus vespilloides* is also unable to induce immune genes in the egg, which explains the reduced survival of their eggs when exposed to bacteria (Jacobs et al., 2014b). From these and other studies, it is clear that there is much to learn about endogenous egg defenses. We have previously hypothesized that there should be a trade-off between fast development and endogenous protection in eggs (Jacobs et al., 2014b). Although increased parental investment could also compensate for the lack of an endogenous egg immune response (Boos et al., 2014; Jacobs et al., 2016). It is clear however that endogenous egg defenses are very unlikely to be limited to just a few insect species.

In conclusion, we show that the eggs of *Tenebrio molitor* possess an impressive endogenous immune response. Furthermore, we show that the same genes are induced in adults and that several genes in the eggs reach transcription levels comparable to adults. Although much remains to be learned about endogenous egg defenses and their interplay with *trans*-generational immune priming and parental investment, our data provides new insights into how eggs are able to survive in a world full of microbes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.12.007.

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