Innate immunity, developmental speed and their trade-offs in two hexapod models

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Chapter 2
Immune competence in eggs of the springtail *Orchesella cincta*

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Abstract

The serosa is an extraembryonic epithelium unique to insect eggs. In the beetle *Tribolium castaneum*, this epithelium can mount an immune response protecting the embryo. Here, we expand our studies beyond the insects, and investigate the immune response in eggs of the springtail *Orchesella cincta* belonging to the closely related hexapod subclass Collembola that do not possess a serosa. Although qPCR could not detect significant upregulation of immune genes in adults, we did find significant upregulation of four out of the thirteen investigated antimicrobial peptides in eggs upon challenge with a mix of Gram positive and Gram negative bacteria. In line with the presence of this inducible response in eggs, we found no evidence for maternal provision of antimicrobials to the eggs in zone-of-inhibition assays of egg extracts. We conclude that mothers do not load their eggs with antimicrobials, and that *O. cincta* eggs can upregulate immune genes upon infection, without the requirement of a serosa. The exact tissue expressing these immune genes in the egg remains to be determined.

Introduction

The serosa is an extraembryonic epithelium that covers the yolk and embryo in insect eggs (Panfilio, 2008; Roth, 2004). It is an evolutionary innovation of the insects and is not present in the other arthropods, such as crustaceans and entognatha (Jacobs et al., 2013; Machida and Ando, 1998). As the serosa secretes a cuticle, it protects the egg against desiccation (Hinton, 1981; Jacobs et al., 2013; Rezende et al., 2008; Vargas et al., 2014). Hence, the serosa was probably crucial for the terrestrial radiation of the insects (Jacobs et al., 2013; Zeh et al., 1989).

The serosa also provides the holometabolous beetle *Tribolium castaneum* with a potent, full range immune response. The serosa can express massive amounts of antimicrobial peptides (AMPs) when challenged with a mix of Gram positive and Gram negative bacteria. Bacteria propagate twice as fast in serosa-less eggs (Jacobs et al., 2014a). Induced expression of functional AMPs in the serosa was also recently found in the hemimetabolous insects *Oncopeltus fasciatus* and *Locusta migratoria* (Jacobs et al., 2022). In addition, induction of antimicrobial peptides could be detected in egg fractions that contained the yolk and serosa of the moth *Manduca sexta,* but not in fractions only containing isolated germ bands (Gorman et al., 2004). Thus, the serosa provides the insect egg with an innate immune response.

Strikingly, the eggs of well-studied model organism *Drosophila* do not show an immune response after bacterial infection (Jacobs and van der Zee, 2013). *Drosophila* eggs do not have a serosa, as *Drosophila* belongs to the Schizophoran flies, the only group of insects that secondarily lost the serosa in evolution (Rafiqi et al., 2008; Schmidt-Ott, 2000). Absence of an immune response in Schizophoran flies may be compensated by maternal investments, as maternal antimicrobial peptides have been found in the outer chorion of eggs of the Mediterranean fruit fly *Ceratitis capitata* (Marchini et al., 1997). Thus, the presence of an inducible immune response in the egg correlates with the presence of a serosa, and eggs without a zygotic immune response seem to require maternal protection. Indeed, arthropod groups without a serosa, such as the crustaceans, do large maternal investments in egg protection (Sastry et al., 1983).

To test the hypothesis that the serosa is a prerequisite for an inducible innate immune response, we investigate the immune response in eggs and maternal protection in the springtail *Orchesella cincta*. *O. cincta* is a wingless, soil-dwelling entognathan and is not an insect, but belongs to the closely related hexapod subclass Collembola (springtails) that do not possess a serosa (Anderson, 1973; Jacobs et al., 2013; Machida and Ando, 1998) (Figure 1-7). Consequently, we do not expect an inducible immune response in these eggs. We perform qPCR on 13 predicted AMPs and one metallothionein after bacterial challenge, and use zone-of-inhibition assays to test the presence of maternal antimicrobials. Surprisingly, we did not find a significant immune response in adults. We did, however, find significant upregulation of 4 AMPs in *Orchesella* eggs after infection. Together with the absence of maternal protection, this indicates that *Orchessella* eggs can mount a zygotic immune response and that the serosa is not required for this.

Materials and Methods

Rearing of the springtails and egg collection

The springtails (*Orchesella cincta*) were obtained from the Animal Ecology Department of Vrije University Amsterdam and kept at 80% humidity and 20 degrees Celsius in 16 cm wide plastic container tubes, closed with netting on top and Paris plaster at the bottom, under a photoperiod of 16h light and 8h dark. Wetted twigs from a location without heavy metal or insecticide contamination were provided. The colony was sprayed with tap water three times a week. To obtain eggs, 3 males and 3 females were put in similar 5 cm wide container tubes.

Bacterial challenge experiment

Micrococcus luteus or *Escherichia coli* cultures were grown overnight in LB medium while shaking at 200 rpm at 37°C. Cultures were centrifuged at 2000 rpm for 10 min at room temperature to harvest bacteria, and pellets were mixed 1:1. For infection of adults, three springtails were put in a petri dish and cooled for 3 min on ice. They were then pricked in the abdomen with a tungsten needle that was either sterile (control) or dipped in the bacterial suspension (bacterial prick). Adults stick to the needle and were carefully put into a small container with help of a brush.

For infection of eggs, 300 3-4 day old eggs were selected that showed hair-like protrusions of the blastodermal cuticle and polar caps (Vargas et al., 2021). Similar to the adults, eggs were pricked with a tungsten needle that was either sterile (control) or dipped in the bacterial suspension (bacterial

prick). The eggs were then placed on wetted filter paper in a petri dish. Both the adults and the eggs were incubated for 6 h in the climate room (see materials and methods section 2.1) before RNA extraction. The experiments were performed in three biological replicates (each including control and bacterial prick).

Selection of immune genes and their primers

We found 9 annotated AMPs in the *Orchesella cincta* genome (Faddeeva-Vakhrusheva et al., 2016), and found 7 others by BLAST (see results section 3.1, Table 2-2). Primers were designed using the NCBI primer design tool. Wherever possible, primers were chosen around an intron to avoid amplification from genomic DNA. In addition, we included the stress-induced metallothionein (MT) gene (Timmermans et al., 2005), and used beta-actin as normalizer gene (de Boer et al., 2009). All primers are listed below:

Table 2-1. Primers used for qPCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Orchesella cincta		
Beta-actin	CCGTAAGGATCTGTATGCCAACA	CCAGGGCAGTGATCTCCTTTT
AMP1a	TACGGGAGAAGTTTGCGTGG	CTGTTTGCCCAGGAGGTACA
AMP1b2	CCGAATCAAGGGAGAAGGCT	AGACCACTTGGAACATCGCA
AMP1b3	TGCATACTCCTGGTCACCTTG	CGTGTTTCCACATGGTCCAAC
AMP1b4	TGTCGATTTGCTGGTGAGGG	AGGTCCAACATCGCACTCTC
AMP1b5	TTGCCCACCTTTGCGTAATC	TCACAAAGGTCAGCGGTACG
AMP2a	GTCTCGGAAAATGGGCCAGA	ACGAAGCGTCAATGTCCTGA
AMP2b	TTGCACACGCGAAAGAAGTG	GCAAGAGTGCCTCCTCCATT
Defensin	TTGCAATCGGCATGCTGGAG	CCCTCAAACTTGGGCCAACA
Diapausin1	CTGCTCACTTTGGCTTTCGT	CCTGCAGCATTCGTTGATCTC
Diapausin2	GCTTTCGTTCTGATTGCCACT	AGCCCTGCAGCATTCATTGAT
*MT *)*	GGCAAATCGCCCACTTGTT	CCTTGCAGACACAATCTGGACC
Toxin1	ACACTCCAGTTCAACCCTGC	ATGAGCTTGACAGCACACGA
Toxin2	AGTGCAACCATGCAACAACC	CCCTTCCACCCTTGTAACCG
Toxin3	CCTTTCTTGCAGTCCTCTTTGC	GGAGTGTCTCGGCGTTTGTA

*) *Metallothionein*

RNA extraction and qPCR

Total RNA of was extracted using trizol extraction (Invitrogen) after which the RNA was purified and DNA digested on column with the RNeasy kit (Qiagen). The quality of RNA preparation was confirmed spectro-photometrically. One microgram of total RNA was used for cDNA synthesis. First strand cDNA was synthesized in the Promega Reverse Transcription system (Promega, Madison, USA). Each qPCR mixture (10 µl) contained 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 (Bio-Rad) and a CFX96 thermocycler (Bio-Rad). Thermal cycling conditions used were 95 °C for 30 s, then 40 cycles of 95°C for 15 s, 60°C for 30s, 72°C for 30 s; this was followed by dissociation analysis of a ramp from 65 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). Total RNA for each sample was measured by qPCR twice (technical replication).

Data analysis of qPCR

Fold change upregulation of the immune genes upon bacterial challenge was calculated using the $2^{-\Delta\Delta C}T$ method with the sterile prick samples as calibrator (Livak and Schmittgen, 2001). Statistical difference between the ΔC_T values of the bacterial prick and the sterile prick samples was calculated using T-tests (SPSS software, version 27.0). The level of significance was defined as $P < 0.05$.

Inhibition zone assays

In order to check whether the mother provides antimicrobials to the egg, antimicrobial activity of egg extracts was tested in an inhibition zone assay (Dubuffet et al., 2015; Moret and Schmid-Hempel, 2000) with a few modifications. Briefly, egg extracts was prepared by smashing eggs into an acetic acid solution (0.05%, 100 eggs in 50 µl) followed by centrifuging at 3500 g for 2 min. Supernatants was collected and divided into 5 aliquots, and kept at -20°C. Methanol was used as the other extraction liquid. Bacteria (*E. coil* and *M. luteus*) were cultured overnight and then seeded to 1% LB-agar soft plates at a final concentration of 10^5 microorganisms/ml, respectively. Then, 5 µl 0.05% acetic acid solution with egg extracts was applied on filter discs that were placed on the seeded LB agar plates. Plates were incubated at 37°C for 24 or 48 h according to the tested microorganism after which the diameter of inhibition zones was measured. 5 µl 0.05% acetic acid solution and methanol acted as negative controls. The assays were conducted in triplicate.

Results

Identification of antimicrobial peptides in the *Orchesella cincta* genome

In total, we found 16 potential antimicrobial peptides in the *Orchesella cincta* genome (Table 2-2). Nine of them were annotated as such in the NCBI protein database: One (Ocin01_18400-PA) was annotated as Defensin J1-1; two (Ocin01_05907-PA and Ocin01_07323-PA) were annotated as antimicrobial peptide 1; two (Ocin01_04461-PA and Ocin01_19094-PA) were annotated as antimicrobial peptide 2; two (Ocin01_19250-PA and Ocin01_13871-PA) were annotated as diapause specific protein; and two (Ocin01_16060-PA and Ocin01_13870-PA) were annotated as Toxin-like peptide AaF1CA1. In addition, we found 4 other peptides by blast that were highly similar to antimicrobial peptide 1 (Ocin01_19760-PA, Ocin01_17792-PA, Ocin01_15366-PA and Ocin01_20115-PA). We called them AMP1b2 to AMP1b5. And we found one that was very similar to Toxin-like peptide AaF1CA1 (Ocin01_19251-PA). We called it Toxin3. We also blasted known antimicrobial peptides from other arthropods (Mylonakis et al., 2016) in the *Orchesella* genome, but found no additional potential AMPs, except for two peptides (Ocin01_14538-PA and Ocin01_18172-PA) that were similar to Drosomycin. We did, however, find no expression of these two genes by qPCR. We also did not find any expression of Ocin01_07323-PA (AMP1b1). These genes were excluded for further analysis, but we added the reported stress-induced metallothionein gene (Timmermans et al., 2005).

Table 2-2. Potential antimicrobial peptides in the *Orchesella cincta* genome.

Gene name	NCBI annotation	GenBank	Collembolomics	Remark
Defensin	Defensin J1-1	ODM88282.1	Ocin01_18400-PA	
AMP1a	Antimicrobial peptide 1	ODN00764.1	Ocin01_05907-PA	
AMP1b1	Antimicrobial peptide 1	ODM99353.1	Ocin01_07323-PA	No expression detected by qPCR
AMP1b2	Hypothetical protein	ODM86921.1	Ocin01_19760-PA	Found similar to AMP1b1 by BLAST
AMP1b3	Hypothetical protein	ODM88896.1	Ocin01_17792-PA	Found similar to AMP1b1 by BLAST
AMP1b4	Hypothetical protein	ODM91321.1	Ocin01_15366-PA	Found similar to AMP1b1 by BLAST
AMP1b5	Hypothetical protein	ODM86567.1	Ocin01_20115-PA	Found similar to AMP1b1 by BLAST
AMP2a	Antimicrobial peptide 2	ODN02227.1	Ocin01_04461-PA	
AMP2b	Antimicrobial peptide 2	ODM87588.1	Ocin01_19094-PA	
Diapausin1	Diapause specific peptide	ODM87430.1	Ocin01_19250-PA	
Diapausin2	Diapause specific peptide	ODM92813.1	Ocin01_13871-PA	
Toxin1	Toxin-like peptide AaF1CA1	ODM90622.1	Ocin01_16060-PA	
Toxin2	Toxin-like peptide AaF1CA1	ODM92816.1	Ocin01_13870-PA	
Toxin3	Hypothetical protein	ODM87431.1	Ocin01_19251-PA	Found similar to Toxin2 by BLAST
Drosomycin1	Hypothetical protein	ODM92144.1	Ocin01_14538-PA	No expression detected by qPCR
Drosomycin2	Hypothetical protein	ODM88510.1	Ocin01_18172-PA	No expression detected by qPCR

The expression of potential immune genes in adults and eggs upon bacterial challenge

Surprisingly, we did not find significant upregulation of AMPs upon infection of adults (Figure 2-1). Although some upregulations were on average high (*toxin3* was upregulated 56.1-fold upon infection), this was not significant because of the large spread among the biological replicates. We did, however find significant upregulation of 4 AMPs in eggs, namely of *antimicrobial peptide 1a* (*AMP 1a*, 4.3-fold), *antimicrobial peptide 1b4* (*AMP 1b4*, 5.2-fold), *antimicrobial peptide 2b* (*AMP 2b*, 9.7-fold) and *toxin1* (5.3-fold). The upregulation of *toxin1* in eggs was also significantly higher than in adults. This means that *Orchesella* eggs can upregulate some immune genes upon infection, despite absence of a serosa (Anderson, 1973).

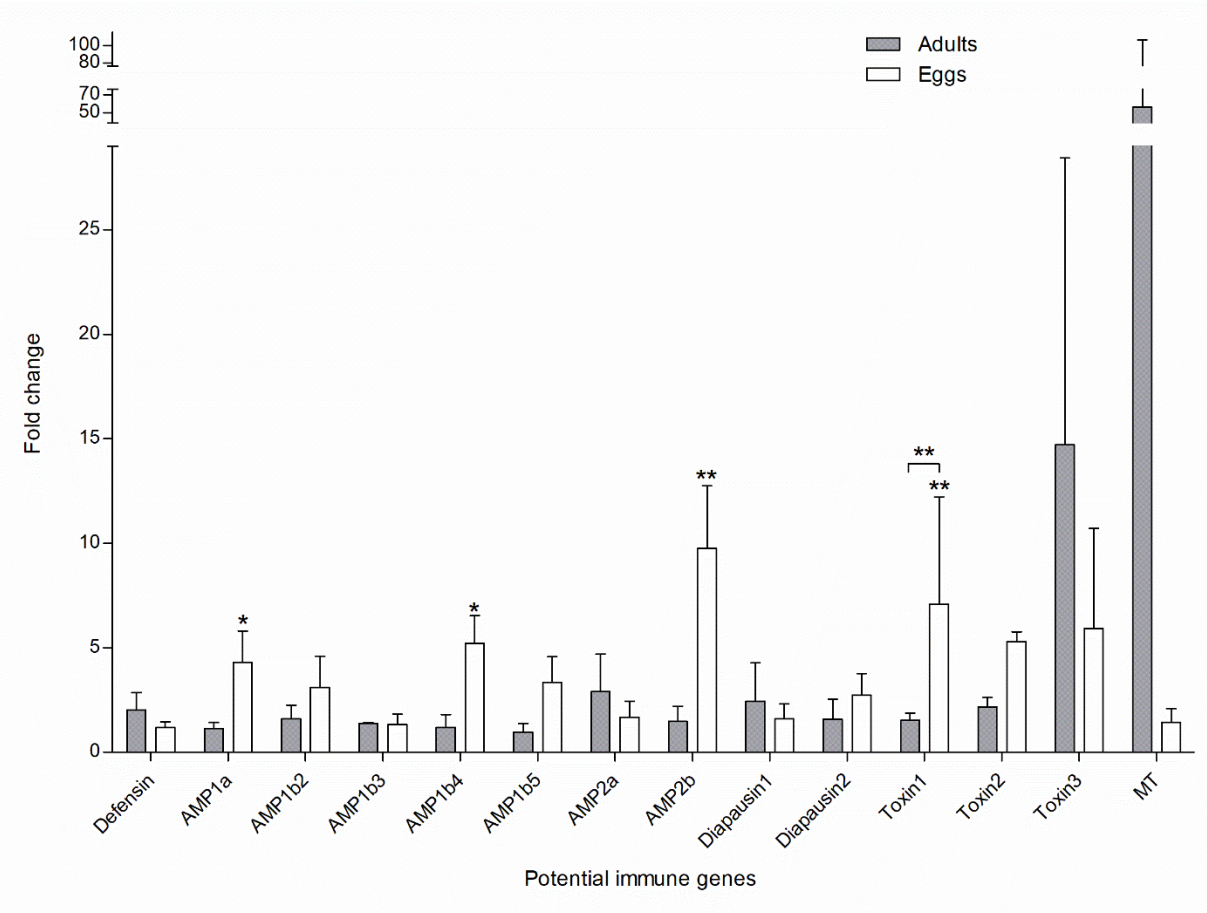

Figure 2-1. Temporal expression of 14 potential immune genes after bacterial challenge. Each vertical bar represented the mean ± S.E (n = 3). Significant difference of fold change of these potential immune genes between adults and eggs was shown with the asterisk above the capped line (* represented $P < 0.05$). Statistical significance of delta Ct between septic injury and sterile injury of each gene in adults or eggs upon injections was indicated with the asterisk above the column (* represented $P < 0.05$, ** represented $P < 0.01$, *** represented $P < 0.001$), shown in Supplementary Figure 2-1. MT: Metallothionein.

Maternally provided antimicrobial activity

To investigate if this immune response eggs is accompanied by standard absence of maternal protection, we investigated antimicrobial activity of eggs laid by unchallenged mothers in zone-of-inhibition assays. Neither water-extracts nor methanol-extracts of eggs prevented growth of the indicator strains *E. coli* or *M. luteus* (Figure 2-2) around discs, whereas clear halo's appeared around control discs with nalidixic acid. This shows that egg extracts of *O. cincta* did not possess antimicrobial activity against the indicator strains, and suggests that *Orchesella* mothers do not load their eggs with antimicrobials.

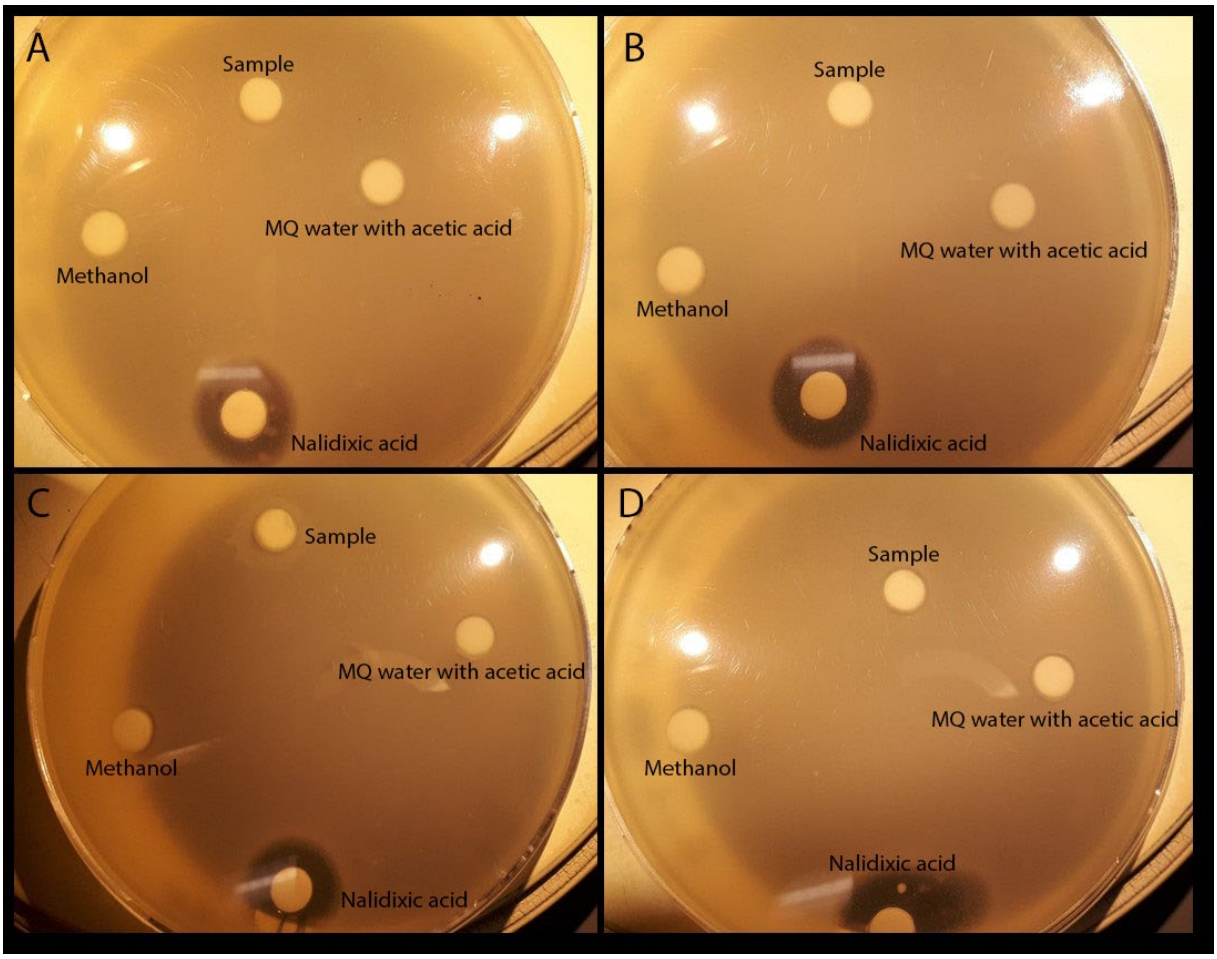

Figure 2-2. Antimicrobial activity of the springtails egg extracts. A: plate with *E. coli* as indicator, the sample was extracted with MQ water (0.05% acetic acid). B: plate with *M. luteus* as indicator, the sample was extracted with MQ water (0.05% acetic acid). C: plate with *E. coli* as indicator, the sample was extracted with methanol. D: plate with *M. luteus* as indicator, the sample was extracted with methanol.

Discussion

Here, we report significant induction of four antimicrobial peptides in eggs of the springtail *Orchesella cincta*. In our study, we could not detect significant induction of AMPs upon infection in adults. As the average upregulation of some immune genes in adults is high (e.g. *Toxin 3* was on average upregulated 63.3-fold upon infection), the lack of significance in adults is due to the large variation among the biological replicates. This large experimental variation may have concealed a true immune response to some extent. In addition, our qPCR approach on candidate genes may be somewhat limited. The thirteen AMPs we tested are predicted AMPs lacking experimental confirmation of their antimicrobial activity. However, several AMP prediction tools, such as AMP_Scanner (Veltri et al., 2018) do suggest antimicrobial activity of the predicted peptides. Finally, other springtails, such as *Folsomia candida* contain bacterial synthesis genes producing the beta-lactam antibiotic (Roelofs et al., 2013). Although beta-lactam genes seem to be absent in *Orchesella* (Suring et al., 2017), we cannot exclude that *Orchesella* adults upregulate other genes upon infection

than the candidate genes tested in this study. Taken together, RNAsequencing after infection may establish a less biased assessment of the immune response of *Orchesella* adults in future.

The most striking finding of our study is the presence of an inducible immune response in *Orchesella* eggs. We did not expect such response, as the immune response in insects is mounted by the extraembryonic serosa (Jacobs et al., 2014a; Jacobs et al., 2022), a structure that is not present in the hexapod subclass Collembola to which *Orchesella* belongs (Anderson, 1973; Jacobs et al., 2013). However, Jura (1972), Machida (2006), and Machida & Ando (1998) call the extraembryonic cells that never envelop the springtail embryo also a serosa (Jura, 1972; Machida, 2006; Machida and Ando, 1998). It is possible that these cells express our detected AMP transcripts, and provide the egg with an innate immune response. It is also possible that embryonic cells express AMPs in *Orchesella*, as immune gene expression has also been reported in late ectodermal cells of *Drosophila melanogaster* and *Oncopeltus fasciatus* embryos (Jacobs et al., 2022; Tan et al., 2014). Future *in situ* hybridizations should reveal the exact location of immune gene expression in *Orchesella cincta* eggs.

Lastly, we did not find any evidence for maternal provision of antibiotics to *Orchesella* eggs. We did, however, only investigate antimicrobial activity of eggs laid by unchallenged mothers. It is possible that mothers only provide antimicrobials to their eggs when challenged. In bumblebees and mealworm beetles, such maternal provision of antimicrobials upon infection has been demonstrated (Moreau et al., 2012; Tetreau et al., 2020). Future analysis of eggs laid by challenged mothers should resolve this issue, but our data demonstrate that *Orchesella* eggs are not standardly loaded with antimicrobials against *E. coli* or *M. luteus* by the mother.

In conclusion, our study shows that *Orchesella cincta* eggs can upregulate immune genes, and that the serosa is not a prerequisite for this. Within insects, the presence of a serosa is also not necessarily coupled to presence of immune competence. Eggs of the beetle *Nicrophorus vespilloides*, for instance, possess a serosa, but lack inducible immune gene expression (Jacobs et al., 2014b). Importantly, even in eggs of other arthropod groups without a serosa, such as the Crustacean *Litopenaeus vannamei*, an upregulation of immune genes has been reported (Alvarez-Lee et al., 2020). Thus, immune competence was probably present in arthropod eggs before the evolution of the serosa. The exact tissue expressing these immune genes in the egg remains to be determined.

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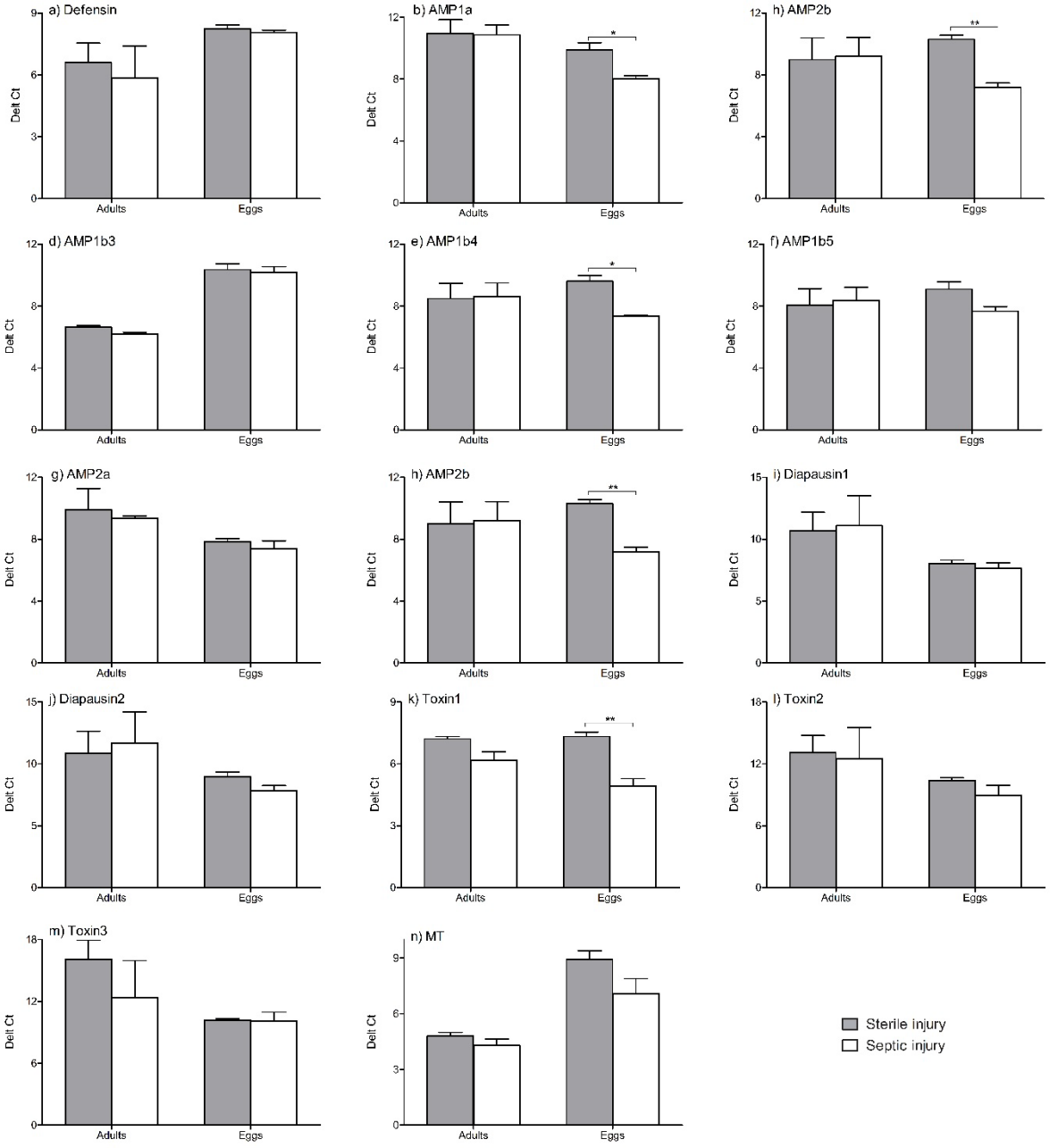
Supplementary information



Supplementary Figure 2-1. Statistical significance of delta Ct between septic injury and sterile injury in adults and eggs upon injections (* represented $P < 0.05$, ** represented $P < 0.01$, *** represented $P < 0.001$). Please notice that y-axis stands for delta Ct: a low delta Ct in septic injury means upregulation of the gene upon bacterial challenge. Each vertical bar represented the mean ± S.E (n = 3). MT: Metallothionein.