Immune function of the serosa in hemimetabolous insect eggs

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Insects comprise more than a million species and many authors have attempted to explain this success by evolutionary innovations. A much overlooked evolutionary novelty of insects is the serosa, an extra-embryonic epithelium around the yolk and embryo. We have shown previously that this epithelium provides innate immune protection to eggs of the beetle Tribolium castaneum. It remained elusive, however, whether this immune competence evolved in the Tribolium lineage or is ancestral to all insects. Here, we expand our studies to two hemimetabolous insects, the bug Oncopeltus fasciatus and the swarming grasshopper Locusta migratoria. For Oncopeltus, RNA sequencing reveals an extensive response upon infection, including the massive upregulation of antimicrobial peptides (AMPs). We demonstrate antimicrobial activity of these peptides using in vitro bacterial growth assays and describe two novel AMP families called Serosins and Ovicins. For both insects, quantitative polymerase chain reaction shows immune competence of the eggs when the serosa is present, and in situ hybridizations demonstrate that immune gene expression is localized in the serosa. This first evidence from hemimetabolous insect eggs suggests that immune competence is an ancestral property of the serosa. The evolutionary origin of the serosa with its immune function might have facilitated the spectacular radiation of the insects.

This article is part of the theme issue ‘Extraembryonic tissues: exploring concepts, definitions and functions across the animal kingdom’.

1. Introduction

Insects are extraordinarily successful and radiated into more than a million species [1]. Over the last century, several authors have attempted to explain the success of insects by the origin of key innovations such as wings or metamorphosis [2,3]. A much overlooked evolutionary novelty, however, is the serosa, an extra-embryonic epithelium in insect eggs that covers the yolk and embryo [4–7]. This epithelium is a synapomorphy of the insects and is not present in other arthropods such as crustaceans [4,5,7,8]. The serosa secretes a cuticle that protects the embryo against desiccation [8–11]. Thus, the origin of the serosa might have opened up a whole new range of terrestrial oviposition sites, facilitating the radiation of insects on land [7,8].

Strikingly, the serosa was secondarily lost in a group of derived flies (the Schizophora) to which the well-studied model insect Drosophila melanogaster belongs [12,13]. This makes the serosa a poorly studied epithelium by evolutionary and developmental biologists. In line with this absence, Drosophila eggs have extremely limited resistance to desiccation [14]. Desiccation is not the only challenge for insect eggs; they are also constantly challenged by pathogens [15–20]. The innate immune response of Drosophila eggs is limited too [21]. It is not until stage 15 (one of the latest developmental stages when the ectoderm and trachea differentiate) that Drosophila eggs show upregulation of antimicrobial peptides (AMPs)
after challenge [22]. Younger eggs cannot contain an infection of non-pathogenic bacteria [22].

In contrast with Drosophila, eggs of the mealworm beetle Tenebrio molitor can mount an extensive innate immune response, including the upregulation of peptidoglycan recognition proteins (PGRPs), Toll and AMPs [23]. These eggs do possess a serosa. Interestingly, inducible AMP expression was also found in the yolk-and-serosa fraction from Manduca sexta eggs, but not in isolated germ bands [24]. We have previously demonstrated that it is the serosal epithelium that harbours the innate immune response in eggs of the beetle Tribolium castaneum [25]. Serosal cells express messenger RNAs (mRNAs) of AMPs, serosa-less eggs do not upregulate immune genes and bacteria propagate twice as fast in serosa-less eggs [25].

It remains unclear, however, whether immune competence of the serosa is ancestral to all insects, or evolved in butterflies and (tenebrionid) beetles. This is particularly elusive, as not only do the serosa-less Drosophila eggs lack an immune response, but so do the serosa-possessing eggs of the burying beetle Nicrophorus vespilloides [17]. In order to address this issue, we expand our studies to the milkweed bug Oncopeltus fasciatus, and the orthopteran Locusta migratoria, both belonging to the Hemimetabola, the basal main group of insects that show incomplete metamorphosis. Upon immune challenge by a Gram-positive and Gram-negative bacterium simultaneously, RNA sequencing reveals an extensive inducible immune response in eggs of the milkweed bug O. fasciatus. We demonstrate the antimicrobial activity of two novel families of AMPs: Serosins and Ovicins. For both the bug and the locust, quantitative polymerase chain reaction (qPCR) shows that eggs are immune responsive when the serosa is present. Using in situ hybridization, we find that transcripts of upregulated AMPs are located in the serosal cells but not in the young embryo of both insects. Our data provide evidence that immune competence is an ancestral feature of the serosa, and we discuss these data in an evolutionary perspective.

2. Material and methods

(a) Insect cultures

Oncopeltus fasciatus were supplied with sunflower seeds and water, and were kept under a 12 h:12 h light:dark cycle at 25°C and 65% relative humidity (RH). Cotton wool was provided for egg lay. Locusta migratoria were ordered from https://www.sprinkhanenwinkel.com/ and kept in cages under a 13 h:11 h light:dark cycle at 32°C and 50% RH. Fresh grass was collected from sprinkhanenwinkel.com/ and kept in cages under a 13 h:11 h light:dark cycle at 25°C and 65% relative humidity (RH). Cotton wool was provided for egg lay.

(b) Infection and RNA isolation

Infection was performed using our previously described standardized infection method [25]. Escherichia coli (DMSZ 10514) and Micrococcus luteus (DMSZ 20030) were grown overnight in liquid Luria-Bertani (LB) broth at 37°C. Optical density was measured and equal numbers of bacteria were spun down for 10 min at 4000g, and the pellets were mixed. Oncopeltus eggs of developmental stages A, B or C (see §2e, quantitative polymerase chain reaction) or 72- to 84-h-old Locusta eggs from at least three different egg cases were then either left naïve, were pricked with a sterile tungsten needle (sterile injury) or were pricked with a tungsten needle that was dipped in this concentrated mixture of bacteria (septic injury). Oncopeltus eggs were kept at 25°C; Locusta eggs at 32°C. Six hours later, RNA was isolated from around 50–100 Oncopeltus eggs, or 6–9 Locusta eggs per treatment using Trizol (Invitrogen) extraction, followed by DNA digestion and column purification (Qiagen RNeasy). RNA quality was confirmed spectrophotometrically and by agarose gel electrophoresis.

(c) Oncopeltus RNA sequencing analysis and genome annotation

Reads were checked for quality using FastQC v. 0.11.5 (http://www.bioinformatics.babraham.ac.uk) and trimmed using Trimomatic v. 0.36; HEADCROP:10 MINLEN:50 TRAILING:20. We assembled the trimmed paired-end RNAseq reads from all samples using Trinity (v. 2.4.0) applying the standard settings [26]. Then, we searched for immune genes in the genome [27] using BLAST and checked gene predictions against our de novo assembly. In total, we modified 47 gene models of Official Gene Set v. 1.2 [28], merged six models into existing ones and added three completely missing models. The full coding sequences of all identified immune genes can be found in the electronic supplementary material, table S1. Next, we mapped all reads to this updated version of the Official Gene Set and genome using hisat (v. 2.0.5) [29,30]. Finally, the counts per gene were calculated using featureCounts (v. 1.5.2), and the data were analysed using DESeq2 [31,32]. A gff and fasta file of all updated gene models, the RNA sequencing data, and details of our expression analysis (R Markdown document) have been deposited in the NCBI’s Gene Expression Omnibus with accession number GSE100429 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100429) [33].

(d) Locusta genome annotation and Locusta quantitative polymerase chain reaction primer design

We searched the available L. migratoria genome https://i5k.nal.usda.gov/data/Arthropoda/locmig−%28Locusta_migratoria%29/ for dorsal and potential AMP sequences using local BLAST [34]. We identified four potential thamautans and eight lysozymes, but could not design qPCR primers for them as sequences were very incomplete. Furthermore, we found dorsal, three Locustins, nine Attacins, six Defensins and four putative defence proteins (that we called ‘PDPs’) showing similarity to Hyphantria cunea Hdd11 [35]. We designed qPCR primers against all of these genes, but detected a reliable qPCR signal only for Locustin1, Attacin3, Attacin7, Defensin1, PDPI−3 and Dorsal. The sequences of used primers can be found in the electronic supplementary material, table S2.

(e) Quantitative polymerase chain reaction

Oncopeltus qPCR primers were designed by Primer3 [36] using genome [27] and our transcriptome, and can be found in the electronic supplementary material, table S2. Oncopeltus complementary DNA (cDNA) was synthesized using the Cloned AMV First Strand Synthesis Kit (Invitrogen); Locusta cDNA was synthesized using the cdNA Synthesis Kit (Promega). A 2.5 ng of cDNA was used in each qPCR reaction. For Oncopeltus, realtime detection was carried out on a CFX96 Thermocycler (Biorad) using the SYBR Green I qPCR core kit (Eurogentec). Thermal conditions were 50°C for 2 min, 95°C for 10 min, 50 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, followed by a ramp from 65 to 95°C to confirm a single-PCR product in a melting curve analysis. In total, qPCR was performed on 27 samples: three treatments (naïve, sterile injury, septic injury) were performed at three stages (stage A, 16- to 24-h-old eggs; stage B, 48–64 h eggs and stage C, 112- to 120 h-old eggs) in
three biological replicates. Each qPCR was done in two technical replicates. From four potential reference genes (Ribosomal Protein L13a, Ribosomal protein 49, actin5c and heat shock protein Hsp90), RPL13a showed the most stable expression levels and was selected as an internal control to calculate ΔCt values. ΔACT values after sterile or septic injury were calculated using the naïve egg samples as calibrator and fold upregulation was then calculated using the 2−ΔΔCt method [37].

For Locusta, qPCRs were performed using the SsoAdvanced Universal SYBR Green Supermix (BioRad) at 95°C for 30 s, 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, followed by a ramp from 65 to 95°C for melting curve analysis (reads every 0.5°C) on six biological replicates, each analysed in two technical replicates. From two potential reference genes (RPL13a and RPL32), RPL32 showed most stable expression and was selected as an internal control to calculate ΔCt. Upregulation after sterile or septic injury was then calculated using the 2−ΔΔCt method with the naïve egg samples as calibrator [37].

(f) In situ hybridization

For Oncopeltus, an approximately 350 bp fragment of the AMP genes was amplified from cDNA of infected eggs using the primers forward/reverse (5’-3’)

Serotonin-1 GTGGACATGCAGTACTGTCTCAG / GGAACATTAAATAATCCTCTACGACAAC

Ovicin-2 CCTGACTACTTCCTTACCAAGATG / GGTCCTGGTCT

Serosin-1 GTGGACATGCAGTACTGTCTCAG / GGAACATTAAATAATCCTCTACGACAAC

defensin-1 GCCGTCTCAAGATGAATCCTGA / AAACAAACATCTGTTGACCTCCTG

For Locusta, a 600 bp fragment of dorsal and a 300 bp fragment of Locustin were amplified from cDNA of infected eggs using the primers forward/reverse (5’-3’)

Locustin1 TGATAATCGAGGACTCGCAG / AGTATTTGTTTGAATATCCGTTTCA

GATAATCCTCTACGACAAC

dorsal GTTGATCAGACCAGCTGTC / ACCAGCTGCCAGTAATCCT

All fragments were cloned into the TOPO II vector (Invitrogen).

Templates for Locusta probes were generated from positive clones (verified by Sanger sequencing) using the M13 primers. Plasmids containing Oncopeltus inserts (verified by analytical digest with PstI or HindIII) were linearized using XhoI (NEB) to generate a template containing in vitro transcription. Digoxigenin-labelled in situ hybridization probes were transcribed in vitro using the SP6 or T7 MEGAscript kit (Ambion) with Roche RNA labelling mix (Roche). For Oncopeltus, embryo fixation (6 h after infection) and in situ hybridization were performed as described by Liu & Kaufman [38]. For Locusta, 72- to 84-h-old infected or naïve Locusta eggs were fixed by perforating them 5–15 times with a tungsten needle and incubating them overnight in 9.75% formaldehyde in phosphate-buffered saline (PBS), as suggested for Schistocerca [39]. Eggs were manually dissected to remove the yolk, often separating the embryo from the serosa. In situ hybridization was essentially performed as described for Schistocerca starting with a refixation [40], but after hybridization, unbound probe was washed away consecutively for 5, 10, 15 and 30 min, four times 1 h, and finally overnight with post-hybridization buffer at 55°C [40]. Sheep anti-digoxigenin-alkaline phosphatase antibody (Roche) was used 1: 5000 in blocking solution [40].

(g) Bacterial growth assays

Predicted mature Oncopeltus peptides (see the electronic supplementary material, table S3) were ordered from Biomatik (Cambridge, Ontario, Canada). Escherichia coli (DMSZ 10514) and Mi. luteus (DMSZ 20030) were diluted to 1 × 106 CFUs ml−1 in PBS. Agar plates were made by mixing 1 ml of this suspension in 10 ml of liquid LB agar. Holes were punched in these agar plates using a sterile cork bore. Ten microliters of a 100 µM peptide solution, an Ampicillin solution (1 mg ml−1 against E. coli; 100 µg ml−1 against Mi. luteus) or sterile water was pipetted in such a hole. Plates were photographed after an overnight incubation at 37°C. The experiment was carried out in triplicate.

3. Results

To assess a potential transcriptional response of O. fasciatus eggs upon infection, we compared the complete transcrisptomes of 48- to 64-h-old eggs that were either left naïve, were steriley injured or were pricked with a needle dipped in a mix of E. coli and Mi. luteus bacteria (septic injury). RNA sequencing of three biological replicates per treatment yielded in total more than 210 million reads of which more than 80% could be mapped to the O. fasciatus genome [41]. Infection induced significant upregulation (greater than 1.3-fold upregulation, adjusted p-value less than 0.05) of 67 genes, of which 48 were also significantly upregulated upon sterile injury (see the electronic supplementary material, tables S4 and S5). Twenty-four of the upregulated genes, listed in table 1, were identified as immune genes based on similarity to D. melanogaster immune genes or by potential antimicrobial properties (see below, [42]). Other upregulated genes include four ankyrin repeat containing proteins, two spinster-like proteins, a takeout-like protein and 13 genes of unknown function or without clear homology to Drosophila genes (see the electronic supplementary material, table S4). The upregulated immune genes comprise genes involved in sensing infection, such as serine proteases; genes involved in signalling, such as the Toll receptor and a PGRl, but mainly include effector genes like potential AMPs, metalloproteinases and laccase (table 1).

A heatmap visualizing the number of reads of all immune genes identified in the genome reveals that some immune genes are constitutively expressed at high levels, such as the recognition genes Gram-negative-binding protein and C-type lectins, and components of the c-Jun N-terminal kinase, immune deficiency and Toll signalling pathways (see the electronic supplementary material, table S6). Interestingly, it is cactus-1 that is expressed at high levels rather than cactus-3 which has been shown to be involved in early dorsoventral patterning [43]. This suggests that subfunctionalization may have taken place among the Oncopeltus cactus paralogues. Upregulation upon infection was mostly detected among the execution genes (see the electronic supplementary material, table S6). Taken together, our RNA sequencing analysis indicates that 48- to 64-h-old Oncopeltus eggs can mount an innate immune response.

Some of the upregulated effector genes (table 1) were straightforward to identify as AMPs, such as OFAS005103, OFAS005104 and OFAS005105 that bear similarity to described Defensins. The OFAS009139 and OFAS009140 sequences, however, did not produce any BLAST hits but in Halyomorpha halys. We call them Ovicin1 and 2, respectively. OFAS013883-OFAS13885 only had similarity to undescribed Hemipteran peptides (in Cimex lectularius, Gerris buenoi, Halyomorpha halys, Lygus hesperus, Rhodnius prolixus and Triatoma infestans). We call them Serosin1–3. All these
Table 1. Infection induces an immune response in 48–64 h Oncopeltus eggs. (Fold upregulation of immune genes after sterile or septic injury based on whole transcriptome analysis in three biological replicates. OFAS numbers refer to O. fasciatus Oncoc. Gene Sci 1.2 [28]. Asterisks indicate level of significance. Potential AMPs were predicted using the prediction software on the APD2 website [42]. Upregulated genes after septic injury consist of 18 (potential) immune response execution genes, and six genes involved in the sensory machinery. * adjusted p-value < 0.05. ** adjusted p-value < 0.01. *** adjusted p-value < 0.001.)

| category          | OFAS  | description          | fold upregulation upon sterile injury | fold upregulation upon septic injury |
|-------------------|-------|----------------------|--------------------------------------|-------------------------------------|
| AMPs/effectors    |       |                      |                                      |                                      |
|                   | 013883| potential AMP Serosin-1 | 1.0                                  | 3173.8***                           |
|                   | 013884| potential AMP Serosin-2 | 7.2                                  | 2606.2***                           |
|                   | 013885| potential AMP Serosin-3 | 1.9                                  | 3994.9***                           |
|                   | 009139| potential AMP Ovicin-1  | 1.8                                  | 1366.4***                           |
|                   | 009140| potential AMP Ovicin-2  | 3.6                                  | 1595.2***                           |
|                   | 005103| Defensin-1             | 8.2                                  | 2035.7***                           |
|                   | 005104| Defensin-2             | 1.0                                  | 160.0***                            |
|                   | 005105| Defensin-3             | 2.5                                  | 1136.6***                           |
|                   | 012870| Oncocin                | 0.7                                  | 108.9***                            |
|                   | 009533| potential AMP          | 0.4                                  | 40.3***                             |
|                   | 006190| potential AMP          | 14.1***                              | 14.7***                             |
|                   | 011944| matrix metalloproteinase| 14.5***                             | 9.7***                              |
|                   | 011945| matrix metalloproteinase| 14.0***                             | 9.6***                              |
|                   | 011306| laccase                | 5.8***                               | 11.9***                             |
|                   | 013043| potential AMP          | 4.8**                                | 5.3**                               |
|                   | 009172| potential AMP          | 4.1***                               | 3.7***                              |
|                   | 004519| potential AMP          | 4.6***                               | 3.5***                              |
|                   | 018039| potential AMP          | 2.6***                               | 2.3***                              |
| sensory           |       |                      |                                      |                                      |
|                   | 006421| Serine protease        | 3.5***                               | 3.5***                              |
|                   | 009025| Serine protease        | 3.4***                               | 4.0***                              |
|                   | 009027| Serine protease        | 3.1***                               | 3.3***                              |
|                   | 006419| Serine protease        | 1.2                                  | 1.6**                               |
|                   | 001578| PGRP                   | 3.1**                                | 3.4***                              |
|                   | 011521| Toll1                  | 1.3*                                 | 1.3*                                |

peptides were predicted to be antimicrobial based on their size, charge, hydrophobicity and proline- or glycine-content [42] (figure 1a). To verify their antimicrobial activity, and to verify the functionality of the detected response in general, we synthesized the predicted mature peptides (electronic supplementary material, table S3) and tested their effect on bacterial growth in vitro. Ten microliters of a 100 µM peptide solution was pipetted into wells that were punched in an LB agar plate containing 1 × 10^5 CFUs ml^-1 of E. coli or Mi. luteus. After an overnight incubation at 37°C, no zones of inhibitions were observed around wells with Serosin-1 or Ovicin-2, but clear zones of inhibition demonstrated that Serosin-2 and Defensin-1 are active against E. coli, and that Ovicin-1 and Serosin-3 are active against Mi. luteus (figure 1b). It is striking that the very similar Serosin-2 and Serosin-3 have such different antimicrobial specificity in this assay. One of the other upregulated effector genes, OFAS012870, is probably the previously described AMP Oncocin that is active against Gram-negative human pathogens such as Pseudomonas aeruginosa and Acinetobacter baumannii [44]. Overall, our bacterial growth assay confirms the functionality of the innate immune reaction of Oncopeltus eggs.

To determine when Oncopeltus eggs gain this functional response, we performed qPCR for the six most strongly upregulated AMPs at three developmental stages 6 h after sterile or septic injury. We chose stage A (16–24 h) when the serosa has not yet developed; stage B (48–64 h) when the serosa has enveloped the yolk and embryo and stage C (112–120 h) when the serosa has retracted during katatrepsis [5,45] (figure 2, schematic drawings at the bottom). At the blastoderm stage, no transcriptional upregulation of AMPs was detected upon infection. By contrast, we detected strong upregulation of AMPs at stage B (figure 2). ANOVA analyses with post hoc Tukey HSD reveal that upregulation after infection at stage B is significantly higher than at stage A, but not significantly different from stage C. This confirms that Oncopeltus eggs become immune responsive when the serosa has developed. However, the eggs remain immune responsive after the serosa has disappeared (stage C, figure 2).

To reveal what tissue expresses the AMPs, we performed in situ hybridization for Serosin-1, Ovicin-2 and Defensin-1 at stages B and C after infection. We were unable to detect any expression of Ovicin-2 by in situ hybridization at any stage. By contrast, we found strong expression of Serosin and...
Defensin in the serosal cells at stage B, but not in the underlying embryo (figure 3a–f). This indicates that the serosa harbours the innate immune response of 48- to 64 h-old eggs. In stage C eggs, when the serosa has disappeared, we found expression of Serosin-1 in the developing mandibles and maxillae (figure 3g,h). Most conspicuously, expression was found in a row of patches in the dorsal ectoderm of the antennal, labial and thoracic segments, of which expression in the labial segment was strongest (figure 3g, dorsal view). The location of the expression is best demonstrated in a more ventral view of a different embryo (figure 3i). A very similar expression pattern was found for Defensin-1 (figure 3j–l). In addition, the expression of Serosin-1 was found in the tracheae (figure 3i), the tissue that was found to be immune responsive in late Drosophila embryos too [22]. These expression patterns were not found after sterile injury or using a sense control in situ probe. We conclude that the serosa provides the Oncopeltus egg with an innate immune response until the ectoderm of the embryo proper becomes immune responsive.

Finally, we expanded our studies to the swarming grasshopper L. migratoria and evaluated immune competence of 72 to 84 h-old eggs by qPCR. At this stage, the serosa is present [46]. Our designed primers (see Material and methods) did not give any amplification in 10 cases (Locustin-2 and -3, Attacin-1, -2, -4, Defensin-2, -4, -5, -6 and putative defence protein-4), or un reliably high Ct values in all conditions ($C_T$ greater than 37 for Attacin-5, -6, -8 and -9 and Defensin-3). This could mean that these genes are not expressed in eggs, but could also indicate inefficient primer binding owing to genetic variation, since our cDNA was derived from completely different locusts as was the genomic DNA used for genome assembly [47]. Nevertheless, for all AMPs for which we could detect expression, we observed increased expression upon infection, except for Putative defence protein-1 (figure 4a–g). Statistical analysis of the upregulation after the sterile and septic injury revealed significant upregulation upon infection of Locustin-1, Attacin-7 and Putative defense protein-2 (students t-test as data were normally distributed) and Defensin-1 (Wilcoxon rank sum test as data were not normally distributed) (figure 4a–g). This shows that infection can induce the expression of at least some AMPs in the locust egg.

To investigate if the serosa is the immune competent tissue in locust eggs, we first performed in situ hybridization against the NFκB Dorsal, an important transcription factor in the innate immune response [48]. Dorsal is highly expressed in the serosa of Tribolium, which led to the first suggestion of an immune function for this extraembryonic epithelium [49]. In unchallenged Locusta eggs, Dorsal is indeed expressed in the serosa (figure 4i–k) and not in the 3-day-old germ band of these eggs (figure 4l). As in Tribolium, Dorsal mRNA is not significantly upregulated upon infection in the locust (figure 4h), as it is the mere nuclear translocation of the Dorsal protein that induces immune genes upon infection [48]. We generated an in situ hybridization probe of sufficient length against the most significantly upregulated AMP.

![Table of peptides](image1)

| Peptide      | nos amino acids | molecular weight | net charge | hydrophobic ratio | glycine content | proline content |
|--------------|----------------|------------------|------------|------------------|----------------|----------------|
| Serosin-1    | 69             | 7769.50 Da       | +5         | 23%              | 15%            | 2%             |
| Serosin-2    | 69             | 7671.48 Da       | +6         | 24%              | 14%            | 2%             |
| Serosin-3    | 69             | 7694.43 Da       | +4         | 23%              | 15%            | 2%             |
| Ovicin-1     | 26             | 3134.80 Da       | +7         | 19%              | 7%             | 19%            |
| Ovicin-2     | 22             | 2748.38 Da       | +4         | 30%              | 4%             | 21%            |
| Defensin-1   | 43             | 4743.44 Da       | +4         | 41%              | 6%             | 2%             |

![Figure 1](image2)

**Figure 1.** Analysis of novel potential antimicrobial Oncopeltus peptides. (a) Biochemical properties of Defensin-1 (OFAS005103) and five completely novel potential AMPs predicted by software on the APD2 website [42]. These peptides form two groups (called Serosins and Ovicins) of similar amino acid sequences encoded adjacent on the same strand of a chromosome. (b) Zone-of-inhibition assay. One nanomole of synthesized peptide was applied to holes in an agar plate containing $1 \times 10^6$ CFUs of either Escherichia coli (DMSZ 10514) or Micrococcus luteus (DMSZ 20030) per ml. Sterile water was used as negative control, and Ampicillin as positive control (1 mg ml$^{-1}$ against E. coli and 100 µg ml$^{-1}$ against M. luteus). Clear zones of inhibition were detected around Serosin-2 and Defensin-1 on plates containing Gram-negative bacteria, and around Serosin-3 and Ovicin-1 on plates containing Gram-positive bacteria. (Online version in colour.)
Locustin-1 and observed no expression in unchallenged eggs, but clear expression around the septic injury in the serosa of challenged eggs (figure 4m–o). No specific staining was observed using Dorsal or Locustin sense control probes. The constitutive expression of Dorsal and induced expression of Locustin in the serosa strongly suggest that the serosa is the immune competent tissue in L. migratoria eggs.

Summarizing, qPCRs demonstrated an inducible immune response in eggs of the milkweed bug O. fasciatus and the grasshopper L. migratoria. In situ hybridizations revealed that immune genes are not expressed in the young embryo, but in the serosa. We conclude that the serosa provides immune protection to these insect eggs until the ectoderm of the embryo itself becomes immune competent.
4. Discussion

We have provided, to our knowledge, the first evidence of an immune function for the serosal epithelium in eggs of two hemimetabolous insects. Together with the evidence from the holometabolous insects *Tr. castaneum* and *Ma. sexta* [24,25], this strongly suggests that immune competence is an ancestral feature of the serosa. Interestingly, immune
competence of young eggs has been lost a few times in insect evolution. Eggs of the beetle *N. vespilloides*, for instance, do not mount an innate immune response upon infection, although they do possess a serosa [17]. This might have been driven by a trade-off with developmental speed. *Nicrophorus* eggs develop within 2.5 days at 20°C which is exceptional among beetles [50]. Trade-offs between growth and immune competence are known from plants, birds and insects [51–53]. The loss of immune competence in *Nicrophorus* eggs might have been compensated by maternally provided lysozymes [54].

A similar trade-off with developmental speed might have taken place in *Drosophila*. *Drosophila melanogaster* lacks a serosa [12,13] and early immune competence [21,22]. Embryogenesis takes only 24 h at 25°C, and the first instar larva already is immune competent shortly before hatching [55], probably greatly reducing the requirement of extensive egg protection. In *Ceratitis capitata*, another fruit fly without a serosa, maternal AMPs have been found on the chorion of the eggs [56]. Thus, the loss of immune competence in insect eggs may be compensated by maternal investments. Traditionally, studies on egg protection have focused on these parental investments (e.g. [56–58]). The well-studied social insects, for instance, provide extensive care for their eggs, protecting them against pathogens [59,60]. In general, however, parental care is expensive and only arose in insects

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**Figure 4.** qPCR and *in situ* hybridization of immune gene expression in *L. migratoria* eggs. *(a–h)* Fold upregulation of immune genes 6 h after sterile or septic injury of 72 to 84 h-old eggs compared to same-aged naïve eggs. Light grey bars: sterile injury. Dark grey bars: septic injury. Error bars show s.e. among six biological replicates (each biological replicate value is the mean of two technical replicate values). Upregulation after septic injury compared to sterile injury in locust eggs is *(a) highly significant for Locustin *(p = 0.0004, t₁₀ = −5.24), (b) not significant for Attacin-3 *(p = 0.07, t₀ = −2.01), (c) significant for Attacin-7 *(p = 0.02, t₀ = −2.760), (d) significant for Defensin-1 *(p = 0.02, W = 0), (e) not significant for Putative defence protein-1 *(p = 0.82, t₀ = 0.24), *(f) significant for Putative defence protein-2 *(p = 0.01, t₀ = −3.12), (g) not significant for Putative defence protein-3 *(p = 0.07, t₀ = −2.07), (h) not significant for Dorsal *(p = 0.391, t₀ = −0.96097), *(i–l) In situ hybridization for dorsal in unchallenged 72 to 84 h-old eggs. *(i) The serosa shows constitutive expression of dorsal. *(j) DAPI counterstaining of *(i) shows the serosal nuclei. *(k) Overlay of *(i) and *(j) DAPI signal is quenched in a few instances by the *in situ* signal. *(l) 72 to 84 h-old germ band does not show expression of dorsal. *(m–p) In situ hybridization for Locustin-1 6 h after septic injury of 72 to 84 h-old eggs. *(m) Strong expression of Locustin-1 (dark purple) is visible in the serosa around the septic injury (brown melanization). *(n) DAPI counterstaining of *(m) shows the serosal nuclei. *(o) Overlay of *(m) and *(n). *(p) The germ band of challenged eggs does not show expression of Locustin-1. (Online version in colour.)
in the presence of intense selective pressures and specific behavioural precursors [61]. Indeed, recent evidence in the social termite rather suggests zygotic, endogenous protection, since antifungal activity of eggs arose from within the chorion and increased over developmental time [62]. Maternal mRNAs seem too short-lived to explain this increase of antimicrobial activity [63].

Along the same lines, enhanced survival of eggs in transgenerational immune priming (TGIP) has traditionally been attributed to a maternal investment by loading of antimicrobials into the egg (e.g. [64–66]). In a comprehensive approach, such maternal loading was recently confirmed for *Tc. molitor* [67]. However, the role of a zygotic endogenous response in eggs is currently explicitly considered in TGIP studies (see [68,69] for excellent reviews). Increasing antimicrobial activity over developmental time [70] and enhanced immune gene expression [71] upon parasitism in primed *Ma. sexta* eggs, for instance, suggest a zygotic response. Moreover, a series of papers has identified a mechanism that involves the binding of pathogen-associated molecules to vitellogenin [72], enabling the transfer of specific elicitors from the mother to the egg to induce a zygotic, endogenous response in these eggs [73–75].

The serosa is well suited to provide such zygotic, endogenous immune protection to the egg. The polypliod nuclei of the serosal cells allow the massive synthesis of peptides. The separation of the serosal cells from the embryo proper is the first visible differentiation step in the blastoderm of insects [76]. The serosa can thus protect the embryo long before the ectoderm starts to differentiate. The evolutionary origin of the serosa might have dramatically altered the costs for egg protection. Whereas crustaceans generally carry their eggs with them and make a large maternal investment [77], the serosal cells are of zygotic origin. Crustacean eggs have been reported to upregulate immune genes upon infection, but this upregulation seems to be rather weak (less than fourfold upon infection maximally) and shortly before hatching [78]. The serosa has allowed insects to lay desiccation resistant and strongly immune competent eggs, probably relieving the need for parental care. Thus, the origin of the extraembryonic serosa must have opened up a whole new range of oviposition sites for insects and may have contributed to their spectacular radiation as a Pancrustacean group.

In conclusion, we have provided evidence of immune competence in the serosal epithelium of hemimetabolous insect eggs. This suggests that immune competence is an ancestral character of the serosa. The origin of the immune competent serosa might have greatly facilitated the success of the insects.

**Data accessibility.** The data are provided in the electronic supplementary material [79].

**Conflict of interest declaration.** We declare we have no competing interests.

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