Wolbachia transinfections in Culex quinquefasciatus generate cytoplasmic incompatibility

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Abstract

Culex quinquefasciatus is an important mosquito vector of a number of viral and protozoan pathogens of humans and animals, and naturally carries the endosymbiont Wolbachia pipiensis, strain wPip. Wolbachia are used in two distinct vector control strategies: firstly, population suppression caused by mating incompatibilities between mass-released transfected males and wild females; and secondly, the spread of pathogen transmission-blocking strains through populations. Using embryonic microinjection, two novel Wolbachia transinfections were generated in C. quinquefasciatus using strains native to the mosquito Aedes albopictus: a wAlB single infection, and a wPip plus wAlB superinfection. The wAlB infection showed full bidirectional cytoplasmic incompatibility (CI) with wild-type C. quinquefasciatus in reciprocal crosses. The wPipwAlBA superinfection showed complete unidirectional CI, and therefore population invasibility potential. Whereas the wAlB strain showed comparatively low overall densities, similar to the native wPip, the wPipwAlBA superinfection reached over 400-fold higher densities in the salivary glands compared to the native wPip, suggesting it may be a candidate for pathogen transmission blocking.

Keywords: Culex quinquefasciatus, Wolbachia, cytoplasmic incompatibility, incompatible insect technique, population replacement, transinfection.

Introduction

Culex quinquefasciatus (Say), the southern house mosquito, transmits a number of important human and animal pathogens, including arboviruses such as West Nile and Rift Valley fever, and the filarial nematode Wuchereria bancrofti (Sudomo et al., 2010). It is also significant from the perspective of wildlife conservation, as it transmits avian malaria (Plasmodium relictum) and avian pox virus on the Hawaiian Islands, where it has been incriminated in the decline of several endangered bird species (Van Riper et al., 1986). C. quinquefasciatus exhibits plasticity in host choice, frequently biting humans and other mammals as well as birds, and as such has the potential to act as a bridge vector for zoonotic pathogens (Farajollahi et al., 2011). As a cosmopolitan species, it has a wide distribution throughout the tropics and subtropics where it is frequently associated with urban areas. The larval stages thrive in domestic water bodies polluted with organic matter, such as pit latrines, blocked drainage ditches, and shallow wells. Vector control is generally limited to insecticide treatments and larval-source management. Owing to predominantly night-time biting and indoor resting, the distribution of insecticide-treated nets and the use of indoor residual spraying for the control of malaria-transmitting Anopheles species has applied concomitant selection on C. quinquefasciatus populations, with high levels of insecticide resistance reported in Africa (Norris and Norris, 2011; Jones et al., 2012; Yadouléton et al., 2015) and Asia (Yanola et al., 2015).

C. quinquefasciatus is a member of the Culex pipiens species complex, almost all populations of which are infected at close to 100% frequency with the maternally inherited intracellular endosymbiont Wolbachia pipiensis, strain wPip. Wolbachia is widespread throughout the phyllum Arthropoda, where different strains induce a variety of reproductive manipulations to facilitate host population invasion. A common variant found in mosquitoes and other Diptera is a modification of the infected male germline that results in sterility unless a compensatory Wolbachia-secreted rescue factor is present in the germline of infected females. This coupling of cytoplasmic incompatibility...
(CI) rescue with maternal transmission results in a relative reproductive advantage for Wolbachia-infected females, providing a population invasion potential, with frequency thresholds for spread largely determined by the balance between the positive fitness effects of CI and negative effects on life-history traits (Hancock et al., 2011; Hancock et al., 2016). In the C. pipiens species group, strain wPip induces a particularly complex pattern of crossing types between populations, with both unidirectional and bidirectional CI observed at varying levels of penetrance (Barr, 1980; Magnin et al., 1987; O’Neill and Paterson, 1992; Guillemaud et al., 1997; Sinkins et al., 2005; Walker et al., 2009; Bonneau et al., 2018).

CI provides a mechanism of sterility that can be used to reduce the reproductive potential of a population through the mass-release of males (Laven, 1967; Dobson et al., 2002; Zabalou et al., 2004; Atyame et al., 2011; Calvitti et al., 2012; Chen et al., 2013; Atyame et al., 2016); the development of highly efficient automated sex separation technology makes this feasible on a large scale (Gilbert and Melton, 2018). The natural incompatibilities between wPip variants within the complex could in theory be utilized for sterile male releases; however, it would be highly desirable for practical purposes to select a single ‘universal’ line adapted to mass rearing that generates sterility with the females of all target populations. To do so, it will be necessary to create transinfections with Wolbachia strains introduced from other host species.

Wolbachia has also been shown to possess a strong pathogen-blocking capacity when some novel Wolbachia–host combinations are generated (Moreira et al., 2009; Bian et al., 2010; Kambris et al., 2010; Walker et al., 2011; Blagrove et al., 2012; Ant et al., 2018). Aedes aegypti transinfected with the wAlbB Wolbachia strain, for example, show strong transmission blocking of an arbovirus (Bian et al., 2010; Ant et al., 2018), including dengue, whereas wAlbB-transinfected Anopheles stephensi show reduced Plasmodium falciparum oocyst and sporozoite loads (Bian et al., 2013). Artificial germline transinfection with Wolbachia has so far been limited to Aedes aegypti (Xi et al., 2005; Moreira et al., 2009; Walker et al., 2011; Blagrove et al., 2012), Aedes albopictus (Blagrove et al., 2012; Ant and Sinkins, 2018) and Anopheles stephensi (Bian et al., 2013). The extension of Wolbachia transinfection generation to Culex or other vector species, to allow the exploration of either transmission blocking for replacement strategies or the generation of sterile males for suppression, has been encumbered by the technical challenges inherent in generating stable infections in the laboratory. Here we report the generation of two novel transinfections in C. quinquefasciatus with Wolbachia strains native to Ae. albopictus, including a native-plus-novel strain superinfection. The relative densities achieved by the transinfections, CI crossing patterns, the effects of the novel strains on host fecundity and immune gene expression are presented.

### Results

**Generation of wAlbB and wPipwAlbA lines in C. quinquefasciatus**

A Wolbachia-free C. quinquefasciatus line PelU was previously created by antibiotic treatment of a wild-type wPip-carrying Sri Lankan PeIA colony (Pinto et al., 2013). A wAlbB transinfection was generated by transferring cytoplasm from eggs of a wAlbB-carrying Ae. aegypti line to PelU embryos. A total of 420 PelU embryos were microinjected with wAlbB (Table 1). The wAlbB-carrying C. quinquefasciatus line was generated from a single G0 female. Females of the wAlbB line were outcrossed to PelU males for five generations before a stable inbreeding colony was established. Maternal transmission rates of wAlbB when PelU males were crossed to wAlbB females (i.e. in the absence of CI) were found to be 100% from 200 progeny assessed.

A superinfected C. quinquefasciatus line carrying both wAlbA and wPip was established through transfer of cytoplasm from the eggs of wAlbA-transinfected Ae. aegypti to embryos of the PeIA (wild-type wPip-infected) colony. A total of 580 embryos were microinjected with wAlbA (Table 1). The wPipwAlbA line was established from the progeny of a single superinfected G0 female. Females from

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**Table 1.** Microinjection statistics for strain generation. 'Total embryos injected' is the number of Culex quinquefasciatus embryos microinjected with each Wolbachia strain for each of the wPip and PelU lines. 'Total adults emerged' is the number of microinjected embryos surviving to produce adults, with parentheses showing percentage. 'Total positive G0 females' is the number of resulting adult female mosquitoes that were PCR positive for the transinfecting Wolbachia strain, with parentheses showing percentage of females displaying transmission out of total positive G0 females.

<table>
<thead>
<tr>
<th>Wolbachia strain</th>
<th>wAlbB</th>
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<th>wMel</th>
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<td></td>
<td>Aedes aegypti</td>
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<td>PeIU</td>
<td>wPip</td>
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<td>580</td>
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<tr>
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<td>58 (10)</td>
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<tr>
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<td>8</td>
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<tr>
<td>Total G0–G1 maternal transmission (%)</td>
<td>0</td>
<td>2 (11)</td>
<td>2 (25)</td>
</tr>
</tbody>
</table>

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this line were backcrossed for five generations to males of the wPip line before a stable inbreeding colony was established. wPipwAlbA females were crossed to PelU males to evaluate rates of maternal inheritance in the absence of CI. Strain-specific PCR indicated that the superinfection was transmitted at 100% fidelity from 200 progeny assessed.

Attempts were also made to generate a line carrying wMel, a Wolbachia strain native to the fruit fly Drosophila melanogaster. Embryos from a transinfected strain of Ae. aegypti were used as the source of wMel, and although more than 1700 embryos of the wPip and PelU lines were injected, far more than for wAlbA and wAlbB, no stable transinfection was generated (Table 1).

CI crossing patterns and fecundity

Crosses were set up between the transinfected, wPip (wild-type) and PelU lines. No eggs hatched from reciprocal crosses between the wAlbB line and the wPip line, displaying a classical pattern of complete bidirectional CI (Fig. 1). Egg hatch rates from crosses between PelU males and wAlbB females were not significantly different from wild-type hatch rates (p = 0.077, Fisher's exact test), suggesting little effect of wAlbB on embryonic viability.

When males of the wPipwAlbA line were crossed to wPip females no egg hatching was observed, whereas wPip-wAlbA females were fully compatible with wPip males and displayed no reduction in hatch rates compared to wPip within-strain crosses (p = 0.586, Fisher's exact test), suggesting full wPip CI rescue (Fig. 2). The wPipwAlbA line therefore displayed a classical pattern of complete unidirectional CI with wild-type C. quinquefasciatus. Eggs resulting from crosses between females of the wPipwAlbA line and Wolbachia-free males showed similar hatch rates to those seen for the wPip colony (p = 0.238, Fisher's exact test), suggesting little or no negative effects of the Wolbachia superinfection on embryo hatch rates in non-CI crosses.

The effects of Wolbachia infection status on the mean number of eggs produced by a female in an egg raft was assessed. No significant effect of Wolbachia infection status or strain was detected (Fig. 3; p > 0.4 for all comparisons, one-way analysis of variance (ANOVA) with Dunnett's), indicating that the presence of non-native Wolbachia did not result in a reduction in fecundity, at least over the first gonotrophic cycle.

Wolbachia densities

Total Wolbachia densities were measured in 5-day-old whole female carcasses, dissected salivary glands and ovary tissue (Fig. 4). The wAlbB line displayed the lowest whole carcass density, with a mean of 1.64 (±1.11 SD) Wolbachia per host genome copies, significantly lower than the 4.34 (±1.68 SD) Wolbachia per host genome for the native wPip strain (p = 0.014, one-way ANOVA with Dunnett's). The wPipwAlbA superinfection reached a significantly higher density than wild-type with a mean of 13.45 (±6.19 SD) Wolbachia per host genome copies (p = 0.00765, one-way ANOVA with Dunnett's). Densities of

Figure 1. Percentage egg hatching rates from individual egg rafts resulting from crosses between the wild-type Wolbachia wPip, the wAlbB and the Wolbachia-ve (PelU, antibiotic-treated) lines. Boxplots show median values and interquartile ranges. Dots show hatching rates from individual egg rafts.

Figure 2. Percentage egg hatching rates from individual egg rafts resulting from crosses between the wild-type wPip, the wPipwAlbA and the Wolbachia-ve (PelU, antibiotic-treated) lines. Boxplots show median values and interquartile ranges. Dots show hatching rates from individual egg rafts.

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Wolbachia in the ovaries were not found to vary between the transinfected and wild-type line \((p > 0.075\) for both comparisons, one-way ANOVA with Dunnett’s). For the salivary glands, however, a significantly higher mean density was observed for the wPipAlbA superinfection compared to the wPip strain alone \((p < 0.0001\), one-way ANOVA with Dunnett’s), with 200.84 \((\pm 47.31\) SD) compared to 0.494 \((\pm 0.36\) SD) Wolbachia per host genome copies, respectively. The wAlbB strain showed a mean salivary-gland density of 8.59 \((\pm 7.23\) SD) Wolbachia per host genome, a nonsignificant difference compared to wPip \((p = 0.072\), one-way ANOVA with Dunnett’s).

Immune gene expression

The transcription of a selection of immune genes was measured in whole adult females of the wAlbB and wPipwAlbA lines and was compared to transcription levels in wPip females. Immune genes investigated were: Rel1 (a homologue of Drosophila dorsal) and Rel2 (an NF-κB transcriptional factor), regulators of the Toll and IMD pathways respectively, Defensin1, which can be activated through both Toll and Immune deficiency pathway (IMD) signalling, and the leucine-rich repeat immune protein 1 \((LRIM1)\), part of the complement-like pathway. No significant effect of either Wolbachia strain was found on immune gene transcription \((p > 0.2\), one-way ANOVA with Dunnett’s) (Fig. 5).

Discussion

The two novel Wolbachia transinfections in C. quinquefasciatus reported here could potentially contribute to control in two ways: by providing a source of sterile males for population suppression, and through pathogen transmission blocking via population replacement. Males of the wAlbB-only infection and the wPipwAlbA superinfection both caused fully penetrant CI when crossed to wild-type females. Females of the wAlbB line were also incompatible with wild-type males, a bidirectional CI pattern resulting in high invasion thresholds, ideal for a suppression strain. No significant effect of wAlbB was observed on host fecundity. This suggests the line is relatively fit compared to wild-types, an important factor given successful suppression would depend on the mass-rearing and release of large numbers of fit, competitive incompatible males. Females of the wPipwAlbA line were fully compatible with wild-type males. The superinfection is thus expected to have the capacity to invade and establish in wild populations of the same crossing type as the Sri Lankan Pel wild-type line. Although novel Wolbachia transinfections have been
shown to decrease fecundity in some instances (Hoffmann et al., 2014), probably reducing strain invasiveness (Schmidt et al., 2017), no significant effects of the wPip-wAlbA superinfection were found on fecundity – although an impact of the infection on other life-history traits such as longevity cannot be ruled out. wPipwAlbA in C. quinquefasciatus provides a further example of additive CI, with modification and rescue of co-infecting strains expressed independently (Dobson et al., 2004; Joubert et al., 2016). However, additive superinfection CI is not always stable; a Wolbachia triple infection in Ae. albopictus suggested co-infecting strain interaction, affecting densities and CI rescue of co-infecting strains (Ant and Sinkins, 2018). Attempts to generate a wMel infection in C. quinquefasciatus were unsuccessful. The relatively high numbers of positive G_0 females generated with no resulting G_0–G_1 transmission suggests that there may be factors limiting the transmissibility of wMel in this species.

Wolbachia intracellular density correlates positively with levels of pathogen inhibition (Lu et al., 2012), although there is considerable between-strain variability in blocking capacity (Martinez et al., 2014; Ant et al., 2018). Surprisingly, we found lower average densities for wAlbB compared to the native wPip infection. This was unexpected as novel transinfections tend to show greater somatic tissue dispersal (McGraw et al., 2002), and thereby higher overall densities than native strains. As somatic infections can have deleterious effects on fitness, co-evolutionary pressures acting on both host and symbiont are expected to favour mechanisms that restrict tissue tropism to the testes and ovaries given CI and transovarial transmission. These factors appear to be strain- and host-specific; the native Wolbachia strains in female Ae. albopictus for example, particularly wAlbA, are largely localized to the ovaries and testes, whereas the non-native wMel can be found at high density in somatic tissues (Ant and Sinkins, 2018). A possible explanation for the low density of wAlbB in C. quinquefasciatus is the close phylogenetic relationship of wAlbB and wPip (Ellegaard et al., 2013), with mechanisms selected to restrict wPip in somatic tissues also functioning with wAlbB. As high densities also tend to result in reduced fitness (Chrostek et al., 2013; Sinkins, 2013; Fraser et al., 2017; Ant et al., 2018), the finding that wAlbB achieves low densities in C. quinquefasciatus suggests that any fitness costs in this line may be minimal, important for mass-rearing and mate competition; however, it does also suggest that there will be limited pathogen inhibition potential.

The wPipwAlbA transinfection was found to have an approximately threefold greater whole carcass density than the wPip-only native infection in the PelA line. This appears to be the result of a greater distribution of Wolbachia in somatic tissues, with a 400-fold higher density observed in the salivary glands. A high wAlbB density is consistent with previous results from a transinfection in Ae. aegypti, where wAlbB was found to reach higher densities than a range of other strains, including wAlbB (Ant et al., 2018). This contrasts with the relative densities of the two strains in the native Ae. albopictus, where wAlbB reaches approximately 10% of the density of wAlbB (Dutton and Sinkins, 2004); again, co-evolutionary pressures have probably selected for reproductive tissue localization in the native host. Experiments carried out in Ae. aegypti showed a low virus inhibition potential for wAlbB against the model arbovirus Semliki Forest virus (Ant et al., 2018) following intrathoracic viral challenges, but it is nevertheless able to block transmission of Zika using oral challenges (Chouin-Carneiro et al., 2019). West Nile and Zika are related flaviviruses, and thus wAlbB may have transmission-blocking potential in Culex.

C. quinquefasciatus is a competent vector for a wide variety of pathogens, ranging from viruses including West Nile and Rift Valley fever, to eukaryotes including the protozoan P. relictum and the filarial nematode Wu. bancrofti. Experimental results from a range of host species suggest that the mechanism of Wolbachia-mediated pathogen inhibition differs between viruses and eukaryotic parasites. Plasmodium and filarial inhibition probably depends at least in part on a priming of the host innate immune system (Kambris et al., 2009; Kambris et al., 2010; Bian et al., 2013). Wolbachia transinfections in Ae. aegypti activate a range of immune signalling pathways, including the Toll, Imd and complement-like pathways (Kambris et al., 2009; Moreira et al., 2009; Rancès et al., 2012). An. gambiae somatically infected with wMelPop block Plasmodium berghei development, which can be restored by knock-down of the Thioester containing protein 1 (TEP1) opsonin (Kambris et al., 2010). No immune priming was detected in the transinfections of C. quinquefasciatus presented here, which included examining defensin, an antimicrobial peptide that was very highly upregulated in wMelPop- and wAlbA-infected Ae. aegypti (Bian et al., 2010; Rancès et al., 2012). This lack of immune upregulation suggests that any blocking of eukaryotic parasites in these Wolbachia transinfections may be limited. In contrast, Wolbachia-mediated blocking of viruses does not appear to require immune priming (Blagrove et al., 2012; Rancès et al., 2012, 2013; Molloy and Sinkins, 2015). Evidence from Ae. aegypti cells infected with wMelPop and challenged with dengue suggest that blocking is the result of disruption of host cell lipid homeostasis and accumulation of cholesterol in lipid droplets (Geoghegan et al., 2017). A previous study investigating the immune priming of a transinfection of wMel in Ae. albopictus also found very low levels of immune gene upregulation (Blagrove et al., 2012; Molloy and Sinkins, 2015), suggesting that the immune response of natively infected species may have an innate desensitization to the presence of Wolbachia.
The demonstration of strong dengue and chikungunya blocking by the high density wMel infection in Ae. albopictus in the absence of immune priming is encouraging for the potential for viral inhibition in the wPipwAlbA C. quinquefasciatus line presented here.

**Experimental procedures**

**Lines and rearing**

The C. quinquefasciatus wild-type was the Pel line originally colonized in Sri Lanka. The Wolbachia-free PelU line was created by antibiotic treatment (Pinto et al., 2013). The source of wAlbA and wAlbB Wolbachia for cytoplasmic transfers was from transinfected Ae. aegypti colonies (Ant et al., 2018). All mosquito colonies were maintained at 27°C and 70% relative humidity with a 12-h light/dark cycle. Larvae were fed tropical fish pellets (Tetramin, Tetra, Melle, Germany) and adults were given access to a sucrose meal ad libitum. Bloodmeals were provided using a Hemotek artificial blood-feeding system (Hemotek, Blackburn, UK) using defibrinated sheep blood (TCS Biosciences, Botolph Claydon, UK). Eggs were collected by providing a bowl of water for oviposition 3–4 days post blood-feeding.

**Transinfection generation**

The wAlbB C. quinquefasciatus line was generated by transferring cytoplasm from wAlbB-infected Ae. aegypti into embryos derived from the PelU colony. The wPipwAlbA superinfection was generated by transferring cytoplasm from wAlbB-infected Ae. aegypti into embryos derived from the wild-type PelA colony. Microinjections were performed using methods described previously (Blagrove et al., 2012) adapted for Culex mosquitoes. Briefly, ~30-min-old egg rafts were collected and individual eggs lined against a damp nitrocellulose membrane fixed to a glass microscope slide. Eggs were briefly dried (~1 min) and covered in Volta-leaf 10s oil (VWR International, Radnor, PA, USA) for injection. Injected eggs were monitored for 24 h, and neonate larvae removed from oil using a fine paint brush and placed in a bowl of water for development. Female G0 survivors were back-crossed to wild-type males, blood-fed and separated individually for oviposition. G1 females were analysed for Wolbachia infection by strain-specific PCR and eggs from Wolbachia-negative G0 females were discarded. Eggs of Wolbachia-positive females were hatched and G1s were assessed for Wolbachia G0–G1 germline transmission. In generating both the wAlbB and wPipwAlbA lines, two separate G0 females with G1 transinfection transmission were derived. As duplicate transinfections carried the same Wolbachia strains in the same host background, only one line of each was carried forward for characterization—in both instances the G3 colony with the greatest number of individuals was chosen. Individual Wolbachia strains were screened using strain-specific primers: 183F + 691R for wPip; wAlbAF + wAlbAR for wAlbA; wAlbBF + wAlbBR for wAlbB. For sequences see Table 2.

**Maternal inheritance, CI crosses, and fecundity**

To assess rates of maternal inheritance, females from the Wolbachia transinfected lines were crossed to PelU males in pools of 30 males and 15 transinfected females. A bloodmeal was provided and egg rafts collected and hatched individually. DNA from a selection of 10 larvae resulting from each egg raft (100 larvae assessed for each line in total) was extracted at the pupal stage and a PCR for Wolbachia was performed.

Rates of CI induction and rescue both with wild-type mosquitoes and between infected lines were assessed by crossing 30 males and 15 females of each line. A bloodmeal was provided and egg rafts collected and hatched individually. Eggs were counted to assess female fecundity. Resulting larvae were counted at the L2-L3 stage to provide hatching rates. Females with no eggs that hatched were dissected to check spermaticae for successful mating. Unmated females were excluded from hatch rate evaluations.

**Density assessment**

For quantitative PCR analysis, genomic DNA was extracted from mosquitoes using phenol/chloroform. Mosquitoes used in density experiments were adults 5 days post pupal eclosion. Genomic DNA was diluted to 100 ng/μl using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). A Bio-Rad CFX-96 real-time PCR detection system was used (Bio-Rad, Hercules, CA, USA) with 2 x SYBR-Green mastermix (Biotool, Houston, TX, USA). Total Wolbachia density was analysed by relative quantification of the Wolbachia surface protein against the mosquito homothorax gene.

**Immune gene expression**

Adult female RNA was extracted from four to five adult mosquitoes using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions. TRIzol-extracted RNA was DNase I treated and purified via standard phenol/chloroform extraction. cDNA synthesis was performed in a total reaction volume of 10 μl, using an iScript cDNA synthesis kit (Bio-Rad). A Bio-Rad CFX-96 real-time PCR detection system was used (Bio-Rad) with 2 x SYBR-Green mastermix (Biotool). Primers Def1-F

<table>
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+ Def1-R, Rel1-F + Rel1-R, Rel2-F + Rel2-R and LRIM1-F + LRIM1-R were used to assess levels of defensin 1, Rel1, Rel2 and LRIM1, respectively. Levels of target RNA sequences were normalized to the 18S ribosomal RNA house-keeping gene using the Pfaffi method. Primer sequences can be found in Table 2.

Acknowledgements

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