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1 # Knockout of *Anopheles stephensi* immune gene LRIM1 by CRISPR-Cas9 reveals
2 # its unexpected role in reproduction and vector competence

3 Ehud Inbar[1], Abraham G. Eappen[1], Robert T. Alford[2], William Reid[1*], Robert A. Harrell[2], Maryam
4 Hosseini[1], Sumana Chakravarty [1], Tao Li[1], B. Kim Lee Sim[1], Peter F. Billingsley[1] and Stephen L. Hoffman[1&]

5 [1]Sanaria Inc. Rockville, MD 20850. [2]Insect Transformation Facility, Institute for Bioscience and
6 Biotechnology Research, University of Maryland, Rockville, MD 20850.

7 [*]Current address: Dept. Veterinary Pathobiology, University of Missouri, Columbia MO 65211.

8 [&]Correspondence and requests for materials should be addressed to Stephen L. Hoffman

9

10 **Abstract**

11 PfSPZ Vaccine against malaria is composed of *Plasmodium falciparum* (Pf) sporozoites (SPZ)
12 manufactured using aseptically reared *Anopheles stephensi* mosquitoes. Immune response
13 genes of *Anopheles* mosquitoes such as Leucin-Rich protein (LRIM1), inhibit *Plasmodium* SPZ
14 development (sporogony) in mosquitoes by supporting melanization and phagocytosis of
15 ookinetes. With the aim of increasing PfSPZ infection intensities, we generated an *A. stephensi*
16 LRIM1 knockout line*, Δaslrim1*, by embryonic genome editing using CRISPR-Cas9. *Δaslrim1*
17 mosquitoes had a significantly increased midgut bacterial load and an altered microbiome
18 composition, including elimination of commensal acetic acid bacteria. The alterations in the
19 microbiome caused increased mosquito mortality and unexpectedly, significantly reduced
20 sporogony. The survival rate of *Δaslrim1* and their ability to support PfSPZ development, were

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1 partially restored by antibiotic treatment of the mosquitoes, and fully restored to baseline
2 when *Δaslrim1* mosquitoes were produced aseptically. Deletion of LRIM1 also affected
3 reproductive capacity: oviposition, fecundity and male fertility were significantly
4 compromised. Attenuation in fecundity was not associated with the altered microbiome. This
5 work demonstrates that LRIM1's regulation of the microbiome has a major impact on vector
6 competence and longevity of *A. stephensi.* Additionally, LRIM1 deletion identified an
7 unexpected role for this gene in fecundity and reduction of sperm transfer by males.

8

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1 **Introduction**

2 Sanaria® PfSPZ vaccines, composed of aseptic, purified, cryopreserved Plasmodium falciparum
3 (Pf) sporozoites (SPZ are produced in aseptically reared *Anopheles stephensi* mosquitoes [1-5].
4 The cost of each dose of vaccine would be reduced significantly if more PfSPZ could be
5 produced per mosquito. When Pf sexual stage parasites are ingested by *Anopheles* spp.
6 mosquitoes, the parasites mate then transform to ookinetes which penetrate the mosquito
7 midgut and form oocysts, which during sporogony give rise to PfSPZ. After migrating to and
8 invading the mosquito salivary glands, PfSPZ are inoculated into humans when the mosquitoes
9 feed (reviewed by [6, 7]). One way to increase PfSPZ production in mosquitoes would be to
10 reduce innate immune responses in the mosquito midgut [8-11] that are thought to inhibit
11 parasite development.

12 *Anopheles* spp. mosquitoes possess innate immune systems that regulate microbial infections,
13 including *Plasmodium* [10, 12-15]. Invasion of the midgut epithelium by *Plasmodium* ookinetes
14 activates a complement-like cascade initiated by a C3-homolog, thioester-containing
15 glycoprotein 1 (TEP1), that circulates in the mosquito hemolymph [8]. Upon ookinete invasion
16 of the midgut epithelium, TEP1 is cleaved by proteolysis. Cleaved TEP1 forms a complex with
17 leucine-rich repeat (LRR) proteins, LRIM1 and APCL1, which is crucial for the stability of the
18 cleaved form of TEP1 while circulating in the hemolymph [9, 11]. The complex binds to the
19 ookinete surface and labels it for melanization and phagocytosis [8, 10, 16, 17]. Knock-down of
20 TEP1 by RNA interference (RNAi) resulted in a 5-fold increase in *P. berghei* (Pb) oocysts in the
21 midguts of susceptible *A. gambiae* and elimination of melanization in a *P. berghei* refractory
22 *A. gambiae* line, L3-5 [8]. Likewise, knock down of LRIM1 or APCL1 by RNAi led to an ~50-fold

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1 increase in oocysts in the midguts of susceptible *A. gambiae* and elimination of melanized
2 parasites in a refractory *A. gambiae* [11].

3 Based on these findings, we hypothesized that deletion of *A*. *stephensi* immune genes would
4 increase PfSPZ infection intensities. We have previously knocked down LRIM1 by RNAi using
5 the UAS-GAL4 system in which LRIM1 dsRNA was endogenously expressed [18].The knock-
6 down resulted in 4-13-fold increase in midgut oocysts and 2-10-fold increase in salivary gland
7 PfSPZ compared to WT [18]. Here, we used CRISPR-Cas9 to generate a stable LRIM1 knockout
8 line (*Δaslrim1*) of *A. stephensi.* The LRIM1 knockout line was dramatically more susceptible
9 than the wild type (WT) to bacterial infection, but not more susceptible to PfSPZ infection. Our
10 embryonic genome editing had an unexpected off-target effect, profoundly reducing the
11 reproductive capacity of the mosquitoes.

12

13 **Results**

14 *Generation of LRIM1 knockout line in Anopheles stephensi, using CRISPR-Cas9*

15 Multiple short guide (sg) RNAs (Table S1) were used to target the first and the second exons on
16 the 5' end of *A. stephensi* LRIM1 (*aslrim1*) gene (Vector base - ASTE000814). The sgRNAs were
17 mixed with recombinant CRISPR-associated protein 9 (Cas9) and injected into 549 *A. stephensi*
18 embryos (Table S2). Nineteen out of the 549 eggs hatched (3.5%), from which, only 4 females
19 and 6 males had developed to G_0 adults. The adults were sorted, and backcrossed *en masse* to
20 WT males and females as appropriate. Females were provided with a blood meal and allowed
21 to lay eggs. Twenty G_1 egg pools were collected of which, 8 groups of larvae were tested by

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1 PCR, using primers flanking the expected editing position (see primers 1 and 2 in figure S1A
2 and primers table S3). Sequence analysis of the PCR products revealed a 13 bp deletion in the
3 5' of exon 2 (nucleotides 942-954), in one group of larvae out of the eight tested (table S2).
4 The adults from the positive group were sorted and outcrossed to WT and after the blood
5 feeding, the females were set to lay eggs individually. Prior to egg laying, a leg was removed
6 from each female, DNA was extracted from each leg and PCR used to screen for females
7 carrying the deletion. The G_2 adults derived from the positive females were then in-crossed
8 and individual females were allowed to lay eggs. Females were collected after egg laying and
9 tested by PCR-sequencing, and G_3 eggs were collected from positive females. Groups of G_3
10 larvae, derived from each of the positive G_2 females, were tested by PCR with primers designed
11 to detect alleles that were homozygous or heterozygous for the deletion (primers 3 and 4 in
12 Figure S1A). Groups of larvae with the highest frequencies of the deletion alleles were
13 continued on and the cycle repeated itself until a homozygous female was found in G_8. The G_9
14 larvae from that female were all homozygous, demonstrating that the null deletion was fixed
15 in the mosquito line. PCR and sequence analysis on individual mosquitoes from G_{12} indicated
16 that the deletion allele remained stable in the population in later generations (Figures S1B and
17 S1C). Importantly, no evidence for the WT LRIM1 allele was seen in any of the mosquitoes
18 tested, indicating the establishment of a stable, homozygous LRIM1 knockout line. This line will
19 be referred to as *Δaslrim1* hereafter.

20 To confirm that the deletion resulted in silenced gene transcription, we performed qPCR on
21 cDNA made from mRNA from different life stages of WT and *Δaslrim1* mosquitoes using two
22 different reactions. In the first reaction the primers were targeting a region on the transcript

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1 that did not include the 13 bp deletion; therefore, this reaction should have amplified the
2 fragment from both the WT and the *Δaslrim1* lines (Figure S2A, primers 5 and 6). In the second
3 reaction, the 3' end of the reverse primer was anchored in the deletion, which should not have
4 resulted in amplification in the *Δaslrim1* line (primers 7 and 8). Approximately 30% decrease in
5 the total abundance of LRIM1 transcript was observed in all tested life stages of *Δaslrim1* when
6 qPCR was done with the first reaction (primers 5 and 6), suggesting that while the transcript
7 was still present, its abundance was somehow affected by the deletion. As expected, there
8 was no amplification of *Δaslrim1* using the second qPCR reaction (primers 7 and 8) in any of
9 the different life stages, indicating that LRIM1 is not expressed in the *Δaslrim1* line. The
10 functional relationship between LRIM1 and TEP1 [17] prompted us to check whether
11 transcription of TEP1 was affected by the 13 bp deletion in LRIM1 and/or its potential loss of
12 function. No significant change was observed in TEP1 transcription TEP1 in all life stages of
13 *Δaslrim1* compared to WT (Fig S2A).

14 The deletion of 13bp from the gene was predicted to cause a frame shift in the amino acid
15 sequence of the protein at positions 57 to 62, which would potentially result in a stop codon,
16 leaving only a small portion (62 amino acids) of the N-terminus of the protein. A polyclonal
17 antiserum was raised against a 20 amino acid peptide in the middle of the LRIM1 protein,
18 starting in position 84 which should not have reacted with protein in *Δaslrim1*. Western blot
19 analysis of hemolymph extracted from both WT and *Δaslrim1* demonstrated clearly that LRIM1
20 protein was not present in the hemolymph of the *Δaslrim1* line (Figure S2B).

21

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1

2 *LRIM1 controls the midgut microflora and thereby longevity of A. stephensi*

3 We investigated the effect of LRIM1 deletion on microbial populations in the mosquito midgut.
4 First, we assessed the general bacterial loads in the mosquitoes using qPCR. DNA was
5 extracted from pools of 10 mosquitoes, both WT and *Δaslrim1,* (5 pools from each line) and
6 bacterial loads were analyzed by real-time PCR using general 16S rDNA primers, targeting
7 variable region 4 [19] (Figure 1A and see primers 13-14 table S3 for primers). Mosquitoes were
8 washed in ethanol and twice in sterile PBS to prevent inclusion of bacteria from the mosquito
9 surface in the extractions. The total bacterial load in non-fed *Δaslrim1* was 52 ± 18-fold higher
10 than in WT. Similar results were observed when using a culture-based approach when colony
11 forming units (CFU) in *Δaslrim1* midguts were approximately 200-fold higher than the CFU in
12 WT midguts (Figure S4E). In the WT mosquitoes, blood feeding led to an increase in total
13 bacterial loads by 21 ± 8-fold. Uptake of blood increased total bacterial load in *Δaslrim1* by 2.6-
14 fold. However, the variability in the bacterial quantity in this line was high and the results
15 between non blood fed and blood fed were not statistically significant.

16 To evaluate the effect of the LRIM1 deletion on the microbial community composition in the
17 mosquitoes, the DNA pools from both lines were assessed by 16S ribosomal RNA (rRNA) gene
18 amplicon sequencing [20], and bacteria identified to genus level. Alpha diversity indices
19 (calculated at a sequencing depth of 12,000 sequences/sample) indicated significantly lower
20 microbial richness in *Δaslrim1* relative to WT (Figure 1B), but this phenomenon did not
21 manifest itself in significantly different Shannon index values due to higher evenness in

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1 *Δaslrim1* samples (Figure S3A,B). Microbial community structure was significantly different
2 between groups, as measured using analysis of similarity (ANOSIM) on Bray-Curtis similarity
3 values (Figure S3C and S3D). The microbial communities in *Δaslrim1* mosquitoes were
4 significantly different as compared to WT, and within-group *Δaslrim1* microbial communities
5 were also more similar to each other than within-group WT communities (Figure S3D). Using
6 DEApp software (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5291987/) DESeq2 analysis
7 showed that 16 taxa were significantly different in abundance between groups, with 15 of
8 them lower in *Δaslrim1* relative to WT (Figure 1C, Table 1). The most reduced bacteria in
9 *Δaslrim1* mosquitoes were from the genera *Asaia* and *Tanticharoenia*, as well as an
10 unidentified genus of *Acetobacteraceae* . Interestingly, *Acetobacteraceae* are Gram negative
11 bacteria that oxidize sugars to acetic acid during fermentation, some of which are known to be
12 insect commensals, specifically of *Anopheles* mosquitoes [21-23] . A heatmap was generated
13 of the 10 most abundant genera in WT mosquitoes and relative abundance compared with
14 *Δaslrim1.* Proteobacterial genera (*Enterobacter*, *Klebsiella*, *Serratia*) and a genus of
15 *Flavobacteriales* (*Elizabethkingia*) were variably abundant in all animals, but were not
16 significantly different between groups (Figure 1C). Again, the absence of the acetic acid
17 bacteria can be clearly observed. Overall, deletion of LRIM1 led to significant alterations in the
18 mosquito midgut microbiome.

19 We noticed high mortality of the *Δaslrim1* adults and hypothesized that this resulted from the
20 high numbers of bacteria. We therefore monitored the mortality of WT and *Δaslrim1*
21 mosquitoes with and without addition of penicillin-streptomycin (PS) in their sucrose meals.
22 Providing 1% but not 0.5% PS eliminated internal bacterial populations from the WT and

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1 *Δaslrim1* mosquitoes [24, 25] and did not compromise their survival and fecundity (Figure S4).
2 With PS treatment, the longevity of *Δaslrim1* mosquitoes increased while there was no effect
3 on WT (Figure 2A). The mosquitoes were blood fed three days after collection. At that time
4 point, over 90% of the WT mosquitoes were still alive whether they fed on regular sucrose or
5 sucrose with PS. The survival of the *Δaslrim1* was only 69 ± 7% on the day of blood feeding.
6 The survival of the *Δaslrim1* was improved (81 ± 4%) when PS was added to the sucrose meal.
7 A big difference in the mortality was observed 6 days after the collection of the mosquitoes (3
8 days post bloodmeal), when only 20 ± 3% of the *Δaslrim1* were still alive compared to 84 ±
9 10% of the WT ($P<0.05$). The survival in *Δaslrim1* was partially rescued by feeding on PS, as 54
10 ± 10% of the mosquitoes were still alive at this point. A dramatic drop in the survival of
11 *Δaslrim1* was observed 8- and 14-days post collection, when only 8 ± 5% and 6 ± 2% of the
12 mosquitoes had survived respectively, compared to 80 ± 9% and 42 ± 7% survival of WT at
13 these time points, respectively ($P<0.05$). Again, a significant, rescue of the survival in *Δaslrim1*
14 was observed 8-and 14- days after collection, with the addition of PS to the sucrose meal. The
15 results suggest a profound role for LRIM1 in determining the longevity of the mosquitoes via
16 controlling their internal microflora.

17

18 *LRIM1 is indirectly important for development of the* Pf *in non-aseptic A. stephensi*

19 The compliment-like cascade, and specifically LRIM1, are considered pivotal in the interactions
20 between *Plasmodium* parasites and mosquito vectors. To assess the effect of the LRIM1
21 knockout on growth and development of Pf, we infected WT and *Δaslrim1* mosquitoes, grown

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1 under normal (non-aseptic) and aseptic conditions, with Pf (Figure 2). Two-three day old WT
2 and *Δaslrim1* adult mosquitoes were maintained non-aseptically on 15% sucrose ± 1% PS for
3 three days, prior to blood feeding. Seven days post blood feeding, midguts were dissected and
4 oocyst infection intensities were assessed (Figure 2B). In WT mosquitoes, the oocyst intensity
5 was 7.9 geometric mean (GM) oocysts/midgut (95% confidence interval (CI) =7.8-17.3), with a
6 prevalence of 90%. Only 6 *Δaslrim1* female adults survived to 7 days post feeding and
7 surprisingly, none were infected. Addition of PS to the sugar meal of the WT mosquitoes
8 significantly increased the infection to 50.5 oocysts/midgut (95% CI = 57-105.3) and prevalence
9 remained unchanged at 90% (Figure 2B). PS partially rescued the infection in *Δaslrim1* with an
10 intensity of 3.2 (95% CI = 2.3-10.6) oocysts/midgut and prevalence of 60%. Due to the high
11 mortality and the modest rescue by PS in *Δaslrim1* mosquitoes, we repeated the experiment
12 starting with more mosquitoes and treating them with PS for their entire adult life, including
13 throughout Pf development (Figure 2C). Oocyst infections were significantly lower in *Δaslrim1*
14 (GM = 2 oocysts/midgut, 95% CI= 2.5-9.2) versus WT (GM = 50.8 oocysts/midgut, 95% CI =
15 52.3-104.8) ($p<0.0001$). Unlike the previous observation (Figure 2B), feeding on PS by WT
16 mosquitoes reduced infection intensity slightly (GM = 32.2 oocysts/midgut, 95% CI = 26.4-61.4)
17 and prevalence remained high (100%). Addition of PS to the sugar meal of *Δaslrim1*
18 mosquitoes again partially recovered the infections (GM = 8.6 oocysts/midgut, 95% CI = 10.5-
19 31.7, prevalence = 73%). In contrast, under aseptic conditions, the infection intensities
20 between WT (GM = 29.6 oocysts/midgut, 95% CI = 27.3-56) and *Δaslrim1* (GM = 29.9
21 oocysts/midgut, 95% CI = 28.9-51.4) were almost identical and the prevalence was 94% for
22 both lines (Figure 2D). A small, non-significant difference was observed in intensities of PfSPZ

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1 infections; 35,824 PfSPZ/mosquito (95% CI = 41085-87442) in the WT versus 11,590
2 PfSPZ/mosquito (95% CI = 25469-56433) in *Δaslrim1* (Figure 2E). The infection prevalence was
3 100% and 95% in WT and *Δaslrim1,* respectively. Overall, under normal growth conditions,
4 oocyst infection intensities in *Δaslrim1* were significantly lower than in WT mosquitoes. This
5 decrease was partially rescued by addition of PS to the sugar meal, and fully rescued when the
6 mosquitoes were grown aseptically.
7

8 *A role for LRIM1 in mosquito fecundity and reproduction*

9 As there was a profound attenuation in the reproductive capacity of *Δaslrim1* compared to
10 their WT counterparts, we examined fecundity (egg production and egg hatching rate) in
11 females and fertility in males. WT and *Δaslrim1* mosquitoes were provided with 15% sucrose ±
12 1% PS from the day of adult emergence. Females were provided with a bloodmeal 1 week post
13 emergence and engorged females were separated into cages. Three days post bloodmeal,
14 females from WT, WT+PS, *Δaslrim1* and *Δaslrim1*+PS, were placed individually into *Drosophila*
15 tubes and allowed to oviposit. The number of females that laid eggs was determined and the
16 eggs were counted in 34-35 tubes collected randomly from each group (Table 2). The majority
17 of WT females had laid eggs, 92% and 95% of the WT and WT+PS females, respectively while
18 only 61% and 55% of the *Δaslrim1* and *Δaslrim1*+PS, respectively, had oviposited. The number
19 of eggs laid by individual *Δaslrim1* females was reduced significantly to 55.7 (95% CI = 47.2-
20 65.8) and 60.9 (95% CI = 54.9-67.6) in the absence and presence of PS, respectively, from WT
21 111.1 (95% CI = 104.2-126.0) and 97.6 (95% CI = 92.3-114.1) in the absence or presence of PS,

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1 respectively (Figure 3A, Table 2). Deletion of LRIM1 also resulted in significant decrease in
2 hatching rates (Figure 3B and Table 2). In WT mosquitoes, the mean egg hatching rate 52.9%
3 (95% CI = 37.5-74.5) and 72.4% (95% CI = 66.7-78.6) in WT and WT + PS, respectively. In
4 *Δaslrim1*, egg hatching was significantly lower (p<0.0001) at 22.8% (95% CI = 13.5-38.7) and
5 25% (95% CI =17.5-36) for *Δaslrim1* and *Δaslrim1* + PS, respectively. Altogether, deletion of
6 LRIM1 resulted in a significant reduction in fecundity in terms of oviposition rate, number of
7 laid eggs per female and egg hatching rate. None of these were rescued by the addition of PS
8 to the sucrose meal, suggesting that low fecundity in *Δaslrim1* was not associated with the
9 increased bacterial loads. To determine whether the low number of eggs was due to a
10 reduction in blood intake by the *Δaslrim1* mosquitoes, females were randomly collected
11 immediately after the bloodmeal and the volume of bloodmeal was determined). The
12 geometric mean volume of bloodmeal taken by WT mosquitoes was 4.6 µL (95% CI =4.2 -5.0 µL)
13 while in *Δaslrim1* females it was only 2.7 µL (95% CI =2.0 - 3.6 µL) µL of blood (1.7-fold less,
14 P<0.0001) (Figure 3C). *Anopheles* mosquitoes concentrate host blood cells and proteins,
15 simultaneously excreting excess salts and water, in a process called prediuresis, which is
16 exemplified by the release of large blood-colored droplets during feeding [26, 27]. During
17 rearing of the mosquitoes, we noticed a dramatic reduction in prediuresis products in Δ*aslrim1*
18 mosquitoes compared to WT. To demonstrate this difference, we transferred 50 WT and
19 Δ*aslrim1* females each to a paper 473 mL paper container and placed a round filter paper at
20 the bottom of the container. After feeding, more prediuresis products were seen on filter
21 papers from the WT containers compared to filter papers from the Δ*aslrim1* containers (Figure
22 3D), indicating that bloodmeal processing is severely disrupted by the LRIM1 deletion. Total

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1 blood meal protein was measured in both lines to determine if the difference in prediuresis
2 was also manifested in lower acquisition of proteins from the bloodmeal (Figure 3D). The
3 geometric mean of blood meal protein contents/ midgut in Δ*aslrim1* was 553 μg
4 protein/midgut (95% CI = 451.8 -676.1 μg protein/midgut) compared to 706 μg protein/midgut
5 (95% CI = 604.0 – 826.5 μg protein/midgut) in the WT. The reduction in blood intake and/or in
6 prediuresis in Δ*aslrim1* did translate to a significant difference in midgut protein contents.
7

8 *LRIM1 has a role in male fertility*

9 The significant decrease in oviposition and hatching rate in *Δaslrim1* mosquitoes could be due
10 to reduced fertility of *Δaslrim1* males. To assess whether the deletion of LRIM1 had an effect
11 on male fertility, WT female pupae were sorted and crossed with either WT (W-W) or *Δaslrim1*
12 (W-L) males. Mosquitoes were reared to adults, females were provided with a bloodmeal, and
13 the number of eggs per female and the proportion of fecund females were determined 1-day
14 post egg laying. The hatch rate was determined 3-days post egg laying. The females that did
15 not lay eggs were dissected and examined microscopically for the presence of eggs in the
16 ovaries and sperm in the spermatheca (Table 3). Bloodmeal size was the same in W-W and W-
17 L females (data not shown), confirming that blood intake was not a factor in oviposition
18 outcome or the number of eggs. In the W-W, 60.3% of the females laid eggs compared to only
19 14.3% in W-L, consistent with the reduced percentage of egg laying females observed
20 previously in *Δaslrim1* (Table 2). The mean fecundity in W-L was moderately but significantly
21 ($P<0.05$) lower than that of W-W cross (89.1 ± 10.5 eggs/female and 115.3 ± 5.2 eggs/female,

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1 respectively) (Figure 4A). An equal proportion of females that did not lay eggs in W-W and W-
2 L, had eggs in the ovaries (47.1% and 46.7%, respectively). Sperms were detected from the
3 spermathecae of 52.9% of females that did not lay eggs in W-W and only 22.2% in W-L. These
4 data suggest that Δ*aslrim1* males have a reduced capacity to inseminate females or that the
5 number of sperm that they deposit in the females is below the detection level. However,
6 *Δaslrim1* and WT males had similar numbers of sperm in their testes (Figure 4B).

7 LRIM1 is expressed in the hemolymph of *A. stephensi* and *A. gambiae* (See figure S2B and
8 [11]), and in the midgut of *A. gambiae*, 24-48 hours following an infected bloodmeal [28]. Due
9 to the effect of LRIM1 deletion on both fecundity and male fertility, we looked for LRIM1
10 expression in the reproductive organs of the mosquitoes. RNA was extracted from pools of
11 male reproductive organs (MRO - testis, male Accessory glands, vas deferens and ejaculatory
12 duct) and ovaries, from 30 sugar-fed 4-6-day old male and female mosquitoes, respectively.
13 The LRIM1 mRNA abundance in those tissues relative to whole mosquitoes was assessed using
14 real-time PCR with LRIM1 specific primers (primers 7 and 8 table S3). LRIM1 mRNA was
15 significantly more abundant in the male reproductive organs compared to whole mosquitoes.
16 Conversely, we did not detect LRIM1 mRNA in the ovaries (Figure 4C). Western blot analysis
17 indicated expression of LRIM1 protein on male reproductive organs from WT but not *Δlrim1*
18 males.

19

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1 **Discussion**

2 We generated LRIM1 knock out (*Δaslrim1*) *A. stephensi* to reduce the innate immune
3 responses in the midgut against Pf ookinetes and oocysts*,* with the aim of improving PfSPZ
4 numbers for manufacturing PfSPZ products. Using CRISPR-Cas9, we were able to generate a
5 deletion in the LRIM1 coding region that completely prevented expression of LRIM1 protein.
6 Deletion of the LRIM1 gene had a profound impact on the quantity and diversity of the
7 mosquito midgut microbiome, increasing total bacterial load and reducing midgut microflora
8 diversity and richness. The *Δaslrim1* mosquitoes were colonized predominantly by known
9 mosquito commensals such as the proteobacteria *Enterobacter*, *Klebsiella*, *Serratia* and
10 *Flavobacteriales,* as well as by *Elizabethkingia* but other classes such as the acetic acid bacteria
11 were lost from the midgut. The removal of the acetic acid bacteria and specifically the *Asaia*
12 genus, is of a particular interest because it is an important commensal bacterium that is highly
13 abundant in *Aedes* and *Anopheles* species, particularly, *A. stephensi* [21, 29-31]. The *A.*
14 *stephensi* abdomen is colonized predominantly by different *Asaia* species which account for
15 41%, 25% and 20% of the total population in the gut, salivary glands and female reproductive
16 system, respectively [29], and 58% of the bacterial population in the male reproductive
17 system. The changes in the mosquito microbiome and the bacterial overgrowth in *Δaslrim1*
18 mosquitoes significantly reduced their survival, indicating the pivotal but probably indirect role
19 of LRIM1 in mosquito longevity.

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1 Contrary to our expectations, PfSPZ infection intensities did not increase in *Δaslrim1*. Under
2 normal rearing conditions, there was a dramatic reduction in PfSPZ intensities which was
3 partially rescued by treating the mosquitoes with antibiotics, and completely rescued when
4 mosquitoes were reared under aseptic conditions. LRIM1 appears crucial for the development
5 of Pf in non-aseptic *A. stephensi*, most likely by regulation of the mosquito microflora.
6 Therefore, establishing infection and completion of the sporogonic cycle by the parasite,
7 depends heavily on the interactions between the parasite and the internal microflora. The
8 concept of parasite-microbiota interactions inside the mosquito is not new and the
9 antiparasitic effect of bacteria has been demonstrated for a variety of *Anopheles* and
10 *Plasmodium* species [32]. Most of these studies suggest that microbiota-related parasite
11 killing is achieved mainly by the stimulation of the mosquito immune response by the bacteria
12 [33-36]. However, the results of our work, specifically the overwhelming increase in bacterial
13 loads in *Δaslrim1* mosquitoes, strongly suggest that even with a complete immune response,
14 competition for nutrients and/or reduced fitness due to increased bacterial load also plays a
15 major part in determining the ability of the parasite to develop optimally in the mosquito.
16 Whether the anti-parasitic effect of the microbiota is through stimulation of the immune
17 response or simply by competition for resources or reduced mosquito fitness, LRIM1 is
18 important in controlling the microbiome and thereby providing the parasite with an
19 environment in which it can flourish.

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21 The decrease in PfSPZ infection intensity following the LRIM1 deletion was contrary to what
22 had been reported by us and by others, showing an increase in either *P. berghei* (Pb) and Pf

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1 oocyst or PfSPZ numbers upon the knock-down of LRIM1 by RNAi [11, 17, 18, 37]. The
2 differences are likely due to the different genetic manipulation approaches that were
3 taken.Unlike in gene knockouts, RNAi does not lead to a complete elimination of the targeted
4 proteins. In line with this, reduced expression through RNAi of a fibrinogen-related protein 1
5 (FREP1) resulted in only partial protein depletion and subsequently, partial reduction in midgut
6 oocyst infections (50%) [38]. In contrast, complete knockout of FREP1 protein by CRISPR-Cas9
7 resulted in a much stronger suppression of the infection (~80%) [37, 39]. Moreover, the knock-
8 down of LRIM1 in *A. stephensi* by RNAi resulted in significant increase in Pf infection intensity
9 [18]. However, in that work, at least 40% of LRIM1 transcript was still present at all life stages
10 tested. Importantly, the knock-down did not affect the bacterial load in the transgenic
11 mosquitoes and this is likely to be the main reason for the differences between the knock-
12 down of LRIM1 and its complete elimination by CRISPR-Cas9, in the present work. LRIM1
13 dsRNA was injected into 1-2-day old adult *A. gambiae*, which were infected a few days later
14 [11, 17], probably too short a time frame for the excessive bacterial burdens to establish
15 compared to the present work, where the LRIM1 deletion was permanent, affecting
16 mosquitoes right from the early stages of development. In summary, while we cannot rule out
17 that LRIM1 and the complement-like system interacts directly with the parasites as suggested
18 previously, the comparable infections in aseptically reared WT and *Δaslrim1* mosquitoes
19 suggest that the effect of LRIM1 on Pf infection is indirect and mediated by the mosquito
20 microbiota.

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1 Deletion of LRIM1 significantly reduced the fecundity and reproduction of the *Δaslrim1*
2 mosquitoes which could not be rescued by addition of antibiotics to the mosquito sucrose
3 meal, suggesting that the role of LRIM1 in these processes was not mediated by the
4 microbiota. This reduction in fecundity maybe explained at least in part by the reduction in
5 blood intake and in prediuresis by *Δlrim1* females. A direct role of LRIM1 in oogenesis is yet to
6 be defined even though there is some evidence for an association between oogenesis and the
7 complement-like system. Vitellogenin (Vg) is a nutrient transporter, essential for delivering
8 digested bloodmeal peptides to the maturing mosquito oocytes [40, 41]. Disruption of Vg
9 resulted in defects in egg development and in parallel, significant reduction in TEP1-mediated
10 ookinete killing [42]. MosGILT, a mosquito saliva protein, is also involved in this process and its
11 knock-down led to a profound impairment in ovarian development that was coupled with
12 significant reduction in TEP1-mediated parasite killing [43]. In another study, deletion of
13 FREP1, a *Plasmodium* agonist, *in A. gambiae,* led to a significant reduction in infection intensity
14 by both *P. berghei* and *P. falciparum* but also had a profound fitness cost in which blood
15 ingestion, fecundity and egg hatching all decreased significantly [39], supporting the premise
16 of association between parasite infection and reproduction capacity.

17

18 In *Anopheles*, oviposition depends heavily on male's ability to inseminate females and fertilize
19 the eggs [44, 45]. Thus, the reduction in egg laying might also be explained by the effect of
20 LRIM1 deletion on the fertility of the males and their ability to fertilize the eggs. Despite the
21 fact that LRIM1 is expressed in the male reproductive organs, sperm counts in *Δaslrim1* males
22 were comparable to those in WT. Therefore, the role of LRIM1 in male fertility is not based on

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1 the ability of the males to produce sperm but rather on the quality of the sperm, the seminal
2 fluids, and/or on the ability of the males to inseminate the females. The involvement of the
3 complement-like system in determining the quality of the sperm was demonstrated previously
4 [46]; in *A. gambiae,* TEP1 binds to the surface of damaged sperm in the testes, labeling them
5 for removal and thereby allowing for a high rate of healthy sperm production. In this context,
6 the supporting role of LRIM1 in stabilizing the active form of TEP1 [9, 17] may also be relevant
7 in maintaining sperm quality.

8 It is interesting to note that acetic acid bacteria and specifically *Asaia*, that were removed from
9 the microflora in *Δaslrim1* mosquitoes, typically populate the reproductive organs of male and
10 female *A. gambiae* and *A. stephensi* [21, 23, 29]. Moreover, in these mosquitoes, *Asaia* is
11 transmitted horizontally from males to females through mating and vertically from the female
12 to the eggs [29, 47]. We do not have evidence for an association between the elimination of
13 bacteria species such as *Asaia* and the low fecundity in the *Δaslrim1*, but it is possible that the
14 regulation of internal microbiota by LRIM1 allows important commensal bacteria such as *Asaia*
15 to successfully colonize the reproductive organs, and in the absence of LRIM1, such regulation
16 may be disrupted to see overgrowth of other species.

17 The unexpected reduction in mosquito fecundity following LRIM1 deletion was independent of
18 the role of LRIM1 in controlling the midgut microbiota, inferring that LRIM1 has an additional,
19 unidentified role in the reproductive capacity of the mosquitoes. This demonstrates the
20 power of precise CRISPR-Cas9 genome editing to reveal novel functions of targeted genes
21 while raising serious concerns about our ability to accurately attribute specific biological
22 functions to a gene. Such off-target effects are important considerations for embryonic gene

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1 editing by CRISPR-Cas9 in other species, including humans, where creation of adverse
2 phenotypes might not be manifested until later in life [48-50].

3 Our goal was to increase PfSPZ yields in *A. stephensi* and thereby improve PfSPZ production by
4 deleting the gene encoding the *A. stephensi* immune deficiency protein, LRIM1. The resultant
5 mutant line, *Δaslrim1,* was significantly more susceptible to bacteria but not to Pf and
6 moreover, was severely compromised in reproductive capacity. Therefore, this line of
7 mosquitoes will not be useful for the manufacturing of PfSPZ vaccines. We are currently
8 testing the possibility of improving PfSPZ yields by deleting other innate immune responses
9 genes.

10

11

12 Table 1. Relative abundance of bacterial genera, *Δaslrim1* vs wild-type.

Taxon (Genus-Level)	Base Mean	Log2 Fold Change	Padj value
Asaia	2581.84	-14.56	6.38E-21
Tanticharoenia	166.62	-12.24	1.15E-09
Caulobacter	6.64	-7.58	5.90E-03
Aquabacterium	3.89	-6.82	1.57E-02
Niabella	2.73	-6.24	3.58E-02
Enterococcus	1.96	-6.09	3.87E-02
Cellvibrio	10.35	-6.08	4.24E-04
Acetobacteraceae	2100.01	-4.91	1.90E-09
Variovorax	21.36	-4.51	5.93E-04
Deinococcus	6.64	-4.28	8.09E-03
Leptothrix	7.61	-3.99	7.73E-03
Sphingobacteriales; env.OPS 17	15.76	-3.27	2.54E-02
Sphingomonas	12.97	-3.02	5.90E-03
Chloroplast	8.09	-2.99	3.32E-02

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Unassigned	82.2	-1.99	3.40E-02
Acetobacter	2.62	4.17	4.25E-02

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1

2 Table 2. Fecundity in wild-type and Δaslrim1 *Anopheles stephensi* grown on 15% sucrose with or
3 without pen strep

Group	Number of females	Proportion of females ovipositing (%)	Geometric mean number of eggs laid per female (95% Confidence interval)	Geometric mean % Hatching (95% Confidence interval)
WT	34	95.2	111.1 (104.2-126)	52.9 (37.5-74.5)
WT + PS	35	84.9	97.6 (92.3-114.1)	72.4 (66.7-78.6)
Δaslrim1	34	61.4	55.7 (53.1-68.7)	22.8 (13.5-38.7)
Δaslrim1 + PS	35	55.4	60.9 (57.1-70.3)	25 (17.5-36)

4

5

6 Table 3. Egg production in WT females and oviposition following a cross with Δaslrim1 or WT males

Cross	#Live males[2]	#Live females		% Females laying eggs	% Females with eggs in the ovaries*	% Females inseminated *
		Feeding[1]	Egg laying[2]			
♂WT X ♀WT	63	43	27	60.3	47.1	52.9
♂*Δaslrim1* X ♀WT	70	33	12	14.3	46.7	22.2

7 * Percentage is calculated from the number of females that did not lay eggs. [1] The numbers were
8 determined at the day of blood feeding. [2] The numbers were determined at the day of egg laying.

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1 **Figure legends**

2

3 **Figure 1. Effect of LRIM1 deletion on the mosquito microflora.** (A) Real-time PCR
4 quantification of the bacterial population densities in the mosquitoes. The reaction used 16S
5 rDNA primers, 515F and 806R (primers 13 and 14, table S3), targeting the bacterial V4 region
6 of the SSU rDNA [51]. The PCR was done on DNA from pools of 10 females from each mosquito
7 line (WT and *Δaslrim1*) before and after blood feeding. The results represent the mean of 5
8 replicates ± SD. Mosquito S7 rDNA was used as housekeeping gene. (B) Alpha diversity richness
9 analysis (to genus) in WT and *Δaslrim1* indicating higher microbial richness in WT relative
10 *Δaslrim1* (Mann-Whitney U, P=0.047). (C) Heatmap of the 10 most abundant microbial taxa
11 across mosquito lines. Depth of sequencing ranged from 12827 to 65,296 sequences/sample
12 (mean=38,682; median=31,785).

13

14 **Figure 2. Effect of deletion of LRIM1 on mosquito longevity and *Plasmodium falciparum***
15 **infection intensity.** (A) Survival of WT and *Δaslrim1 A. stephensi* over a period of 14 days. Each
16 point represents the mean ± SD of survival percentage in three different cages. * p≤0.05. (B-C)
17 Oocyst numbers in non-aseptic mosquitoes. Oocysts were counted by microscopy 7 days post
18 bloodmeal for WT and *Δaslrim1* mosquitoes grown in <u>non-aseptic</u> conditions and fed on 15%
19 sucrose with or without 1% penicillin/streptomycin (PS). The PS was added to the sugar meal
20 for a short period (3- days prior to the bloodmeal) (B) or, throughout the entire adult life of the
21 mosquitoes (C). Each point represents the oocyst number in a single midgut. (B) n=22, 6, 20

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1 and 20 for WT, *Δaslrim1*, WT + PS *and Δaslrim1* + PS, respectively. (C) n=20, 19, 18 and 22 for
2 WT, *Δaslrim1*, WT + PS *and Δaslrim1* + PS, respectively. The results were analyzed in non-
3 parametric, Kruskal-Wallis and Dunn's multiple comparisons tests. (D) The number of
4 oocysts/midgut in WT and *Δaslrim1* mosquitoes grown under aseptic conditions, n=18 and 17
5 for WT and *Δaslrim1*, respectively. The results were analyzed by the non-parametric, Mann
6 Whitney test. (B-D) The results are expressed as a geometric means ±SD. Since some values
7 were zero, a value of 1 was added to the entire dataset to allow calculation of the geometric
8 means. (E) Number of sporozoites/mosquito in mosquitoes grown in aseptic conditions. The
9 numbers of sporozoites were determined by dissecting the salivary glands of the mosquitoes
10 and counting sporozoites from each mosquito by microscopy. The results are expressed as the
11 geometric mean ± SD (n=20 for both groups). The results for panel E were analyzed by the
12 non-parametric, Mann Whitney test. For all panels, NS $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$ ***
13 $p\leq0.001$, ****$p\leq0.0001$.

14

15 **Figure 3. Fecundity in WT and *Δaslrim1* mosquitoes.** (A) Number of eggs/ females. The
16 number of eggs was determined by counting the eggs laid in single *Drosophila* tubes. The
17 results are expressed as the geometric mean of the number of eggs/females ± SD; n=34 for WT
18 and 35 for the other 3 treatments. (B) Percentage of eggs hatching. The hatching was
19 determined by counting the number hatching larvae in the single *Drosophila* tubes by
20 microscopy, 2 days after egg laying. The percentage is of the total number of eggs laid in that
21 tube (panel A). The results are reported as the geometric mean of the percent hatching ± SD;
22 n=34 for WT and 35 for the other 3 treatments. Since some values were zero, a value of 1 was

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1 added to the entire dataset to allow calculation of the geometric means. (A and B) The data
2 were analyzed by Kruskal-Wallis and Dunn's multiple comparisons tests. **** p≤0.0001. (C)
3 The bloodmeal volumes in WT and *Δaslrim1* mosquitoes immediately after blood feeding. The
4 results represent the geometric mean of bloodmeal volumes ± SD; n=20. (D) Remnants of
5 blood on the filter paper after bloodmeal of 50 females, as an indication of prediuresis. (E)
6 Bloodmeal protein contents in midguts of WT and *Δaslrim1* mosquitoes, immediately after
7 bloodmeal. The protein contents were determined using Lowry protein assay. The results
8 represent the geometric mean of midgut protein contents in micrograms ± SD; n=20. (C and E)
9 The results were analyzed using non-parametric, Mann Whitney test. ** P≤0.01,
10 ****P≤0.0001.

11 **Figure 4. Effect of deletion of LRIM1 on fertility in male mosquitoes.** (A) Number of eggs laid
12 in single *Drosophila* tubes by WT females, mated with WT (W-W) or *Δaslrim1* (W-L) males. The
13 results are the mean number ± SD (n=38 and 10 for W-W and W-L, respectively). Results were
14 analyzed by unpaired T-test. (B) The number of sperms/males was determined by dissecting
15 the male testes and accessory glands from each male and counting the number of sperms in
16 hemocytometer under the microscope. The results are presented as the geometric mean ± SD
17 (n= 40 and 31 for WT and *Δaslrim1* respectively). The data were analyzed using non-
18 parametric, Mann Whitney test. Since some values were zero, a value of 1 was added to the
19 entire dataset to allow calculation of the geometric means. (C) Relative abundance of LRIM1
20 mRNA in ovaries (OVA) and male reproductive organs (MRO) of WT mosquitoes was
21 determined by real-time PCR using primers specific to LRIM1 (primers 5 and 6, Table S3). The
22 abundance was normalized to the mosquito S7 rDNA gene (Primers 9 and 10, table S3). The

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1 results in panel C represent two independent experiments with similar results ** P≤0.01. (D)
2 Western blot analysis done on pools of male reproductive organs (MRO) from 30 WT and
3 *Δaslrim1* male mosquitoes. The proteins were transferred to PVDF membrane and reacted
4 with 1:250 rabbit polyclonal anti LRIM1, generated in this work. Betta actin (1:1000) was used
5 as a loading control.

6 **Figure S1. Generation of *Δaslrim1* mosquito line.** (A) PCR strategy for detection of indels in
7 mosquitoes during the CRISPR procedure. Primers 1 and 2 flank the expected deletion site
8 (marked in red) and are used to amplify both the WT and the deletion alleles. The 3' end of
9 primer 3 (13 bp, marked in red) is anchored in the deletion and thus the primer should anneal
10 only to the WT allele. One base pair of the 3' end of primer 4 is anchored upstream to the
11 deletion while the other 17 bp are anchored downstream for the deletion and thus PCR with
12 this primer should only amplify the deletion allele. (B) Alignment showing Homozygous
13 deletion of 13 nucleotides in LRIM1 gene in 10 randomly collected *Δaslrim1* mosquitoes from
14 $G_{12.}$ The alignment was done on sequences generated from PCR done with primers 1 and 2. (C)
15 Diagnostic PCR amplifying the WT allele (upper panel, primers 1 and 3) and the deletion allele
16 (lower panel, primers 1 and 4) in 2 WT and 10 randomly collected G_{12} *Δaslrim1* mosquitoes.

17 **Figure S2. Conformation of LRIM1 knockout at the RNA and protein levels.** (A) Upper panel -
18 Scheme of the real-time PCR strategy. The nucleotide position indicated refers to the position
19 of the primers on the LRIM1 gene (ASTE000814). PCR I was done with Primers 5 and 6 (Table
20 S3) and targets a region downstream to the deletion and therefore should amplify both the
21 WT and the Deletion alleles. PCR II is done with primers 7 and 8 and is aimed to amplify only
22 the WT allele as the 3' end of primer 5 is anchored in the deletion. Lower panel- Real-time PCR

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1 done with the above primer sets. Results are the mean mRNA abundance ± SD of 4 different
2 replicate RNA samples from larvae, pupae and female and male adults. Each sample is a pool
3 of 10 individuals from each of the life stages. The results are indicated as the LRIM1 or TEP1
4 mRNA abundance relative to the housekeeping, S7 rDNA gene (Primers 9 and 10, table S3). (B)
5 Western blot analysis: Hemolymph from 7 female mosquitoes were loaded in each well in the
6 gel and transferred to PVDF membranes. Membranes were reacted with anti 1:250 LRIM1
7 antiserum generated in this work. The expected molecular weight of LRIM1 protein is 58.3
8 kDa. For loading control, membrane was reacted with 1:500 AsTEP1 antiserum (ABBIOTECH #
9 250881).

10

11

12 **Figure S3. Alpha diversity and analysis of similarity in WT and *Δaslrim1* mosquitoes**

13 (A-B) Comparison of alpha diversity. (A) Shannon index (log base e) of microbial communities
14 in WT and *Δaslrim1* mosquitoes. (B) Microbial community evenness. The differences in both
15 measures were not statistically significant (MWU, P=0.4034 and MWU, P=1 in A and B,
16 respectively). Alpha diversity indices were calculated on rarefied datasets (12,000
17 sequences/sample). (C) Metric Multidimensional Scaling (mMDS) plot of Mosquito-associated
18 microbial communities. Analysis was performed at the taxonomic level of genus. Data was
19 log(x+1) transformed, and Bray-Curtis similarity was calculated for all pairwise comparisons.
20 (D) Bray-Curtis similarity values were represented in two dimensions, and 2D stress was 0.08.

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1 Analysis of similarity (ANOSIM) indicated that microbial communities between the two
2 mosquito lines were significantly different (R=0.524, p=0.008).

3 **Figure S4. The effect of Pen Strep addition to the sucrose meal on midgut bacterial loads,**
4 **survival and fecundity.** (A) Survival of WT mosquitoes maintained on 0%, 0.5% and 1% (v/v) of
5 Penicillin-Streptomycin (PS) solution (500 U/mL), diluted in 15% sucrose. The survival
6 Percentage is the number of live mosquitoes in each time point relative to the number of
7 mosquitoes put in the cage on day zero (30). (B) Mean ±SD of the number of eggs per females
8 grown on different PS concentrations. The mosquitoes were provided with bloodmeal 3 days
9 after transferring them to cages and individual mosquitoes were put in *Drosophila* tubes for
10 oviposition 4 days after bloodmeal. Eggs were counted in each tube. (C) The number of larvae
11 in each *Drosophila* tube was determined 1-2 days post oviposition. The results show the % of
12 larvae out of the total number of eggs laid in that particular tube. (B, C) n=4, 5 and 5 for 0%,
13 0.5% and 1% PS, respectively. (D) Colony forming units (CFU) in individual guts of WT
14 mosquitoes in different PS concentrations. Each gut was diluted 10 and 100 times in sterile PBS
15 and the CFU for each gut is the mean between the two dilutions. The results show the mean
16 CFU for different guts (n= 4, 3 and 3 for 0%, 0.5% and 1%, respectively). (E) Colony forming
17 units (CFU) in individual gut of WT and *Δaslrim1* mosquitoes grown with and without 1%PS
18 ($P \leq 0.05$). Each gut was diluted 10, 100 and 1000 times in sterile PBS and the CFU for each gut is
19 the mean between the three dilutions. The results show the mean±SD CFU for different guts
20 (n= 4).

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4 **Materials and Methods**

5 *Mosquito and parasite growth and infection*

6 The mosquitoes used in this study were *Anopheles stephensi* SDA500, reared at the University
7 of Maryland Insect Transformation Facility (ITF) at the Institute for Bioscience and
8 Biotechnology Research (IBBR), using standard conditions (28^{0}C and 75% humidity). For aseptic
9 rearing of mosquitoes, egg laying was induced and eggs were transferred to Sanaria for aseptic
10 production[1]. For infection of mosquitoes, Pf NF54 strain were grown in blood culture for 18-
11 20 days for the development of gametocytes. Mosquitoes were then fed through a membrane
12 with infected blood (5 million gametocytes/mL), as established previously [1, 52, 53].

13 *Mosquito rearing, CRISPR mix and Injection into A. stephensi embryos*

14 CRISPR mix was done based on the protocol of Kistler et al. [25]. Briefly, short-guide (sg) RNAs
15 were generated by PCR amplification of the guides following by in vitro transcription using the
16 Ambion Megascript T7 kit (#AM1334) according to the manufacturer instructions. The RNAs
17 were purified using Megaclear Transcription Clean-Up kit (Thermo Fisher #AM1908). Multiple
18 sgRNAs (40 ng/µL) were mixed with 300ng of recombinant CAS9 (PNA bio - CP01). CRISPR
19 injection mix was injected by standard methods [54, 55] into preblastoderm *A. stephensi*
20 embryos. Embryos were injected between 40 min (TS) and 60 min (TF) post start of embryo

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1 collection, with T0 being the time mosquitoes were added to egging chamber, Ts being
2 average start time of injection, and TF being average time of completion for the last embryo of
3 an injection round. All injections were done at the University of Maryland ITF at IBBR.

4 *DNA and RNA assessments.*

5 Adults or larvae of *A. stephensi* mosquitoes were homogenized using blue pestles and genomic
6 (g)DNA was extracted from the homogenates using Qiagen DNeasy Blood & Tissue Kit
7 (#69506). RNA was extracted from mosquito homogenates using Qiagen RNeasy mini kit
8 (#74106) according to the manufacturer instructions. PCR reactions were done using MyTaq
9 red DNA polymerase (bioline USA #BIO-21108). Complimentary (c)DNAs were synthesized
10 using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher # 4368814) on extracted
11 RNAs. Real-time PCR reactions were done on cDNAs or gDNAs (for assessment of bacterial
12 loads) using SensiFAST™ Real-Time PCR Kits (bioline # BIO-82005).

13

14 *Production of LRIM1 antibody and western blot analysis.*

15 Poly-clonal αLRIM1 was produced by injecting a short peptide (KLH conjugated), corresponding
16 to amino acids 437-456 of A. stephensi LRIM1 protein, into rabbits (done by Envigo
17 Bioproducts). For western blots, mosquito hemolymph was extracted as described previously
18 [11] and male reproductive organs (testes, male accessory glands, *vasa deferentia* and
19 ejaculatory ducts) were each pooled from 30 mosquitoes and lysed in RIPA lysis and extraction
20 buffer (Thermo Scientific #89900) according to the manufacturer instructions. Samples were run on

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1 SDS-PAGE gel and probed with the polyclonal αLRIM1 antiserum at 1:250 dilution, then a 1:5000
2 secondary anti rabbit (AP conjugated).
3
4 *Survival, fecundity bloodmeal volume and protein content.*
5 To estimate mosquitoes' survival, a known number of mosquitoes were separated to a clean
6 gallon container. Dead mosquitoes were removed from the containers at the indicated time
7 points and counted. The number of dead mosquitoes was subtracted from the total number
8 of mosquitoes. To determine egg laying, single blood-fed mosquitoes were removed from the
9 cages and placed in a single *Drosophila* tube containing water and sealed with a cotton ball.
10 Mosquitoes were allowed to lay eggs for 1-2 days in a humidified 28°C room before being
11 removed from the tubes. The eggs were counted under dissection microscope. The *Drosophila*
12 tubes were incubated for an additional 1-2 days in the same conditions and the larvae were
13 counted under dissection microscope. The percentage of larvae hatching was determined per
14 the total number of eggs in the tube. For determination of bloodmeal, midguts were dissected
15 immediately after blood feeding (2 cycles of 20 minutes per cage) and homogenized in 100
16 microliter PBS. A standard curve was made and hemoglobin was determined in
17 spectrophotometer at a wavelength of 412 nM, as was done previously [56]. Similarly,
18 bloodmeal protein contents were determined using the Lowry protein assay kit (Thermo #
19 23240) on midguts dissected immediately after blood feeding.
20
21 *Midgut bacterial loads*

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1 To assess the effect of adding antibiotics to the sucrose meal on the midgut bacterial loads,
2 adult mosquitoes were fed with 15% sucrose with or without different concentrations of
3 Penicillin-streptomycin from the time of emergence. Midguts were dissected and
4 homogenized in 100 microliters of sterile PBS. Serial dilutions of guts homogenates were then
5 plated on LB-agar plates and colonies were counted to determine colony forming units (CFU).

6 *Microbial diversity analyses*

7 Genomic DNA was PCR amplified with primers 515F and 926R [57], targeting the V4 and V5
8 variable regions of the microbial small subunit ribosomal RNA gene using a two-stage
9 “targeted amplicon sequencing (TAS)” protocol as described previously [20]. Primers were
10 modified to include linker sequences at the 5’ ends (i.e., so-called “common sequences” or CS1
11 and CS2 on forward and reverse primers, respectively). First stage PCR amplifications were
12 performed in 10 microliter reactions in 96-well plates, using the MyTaq HS 2X master mix
13 (BioLine, Taunton, MA, USA). PCR conditions were 95°C for 5 minutes, followed by 28 cycles of
14 95°C for 30”, 50°C for 60” and 72°C for 90”. Subsequently, a second PCR amplification was
15 performed in 10 microliter reactions in 96-well plates. A master mix for the entire plate was
16 made using the MyTaq HS 2X master mix. Each well received a separate primer pair with a
17 unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina
18 (Fluidigm, South San Francisco, CA; Item# 100-4876). These AccessArray primers contained the
19 CS1 and CS2 linkers at the 3’ ends of the oligonucleotides. Cycling conditions were as follows:
20 95°C for 5 minutes, followed by 8 cycles of 95°C for 30”, 60°C for 30” and 72°C for 30”. A final,
21 7-minute elongation step was performed at 72°C. Samples were pooled in equal volume using
22 an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library

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1 was purified using an AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-
2 Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-
3 in, were loaded onto an Illumina MiniSeq mid-output flow cell (2x150 paired-end reads). Based
4 on the distribution of reads per barcode, the amplicons (before purification) were re-pooled to
5 generate a more balanced distribution of reads. The re-pooled library was purified using
6 AMPure XP cleanup, as described above. The re-pooled libraries, with a 15% phiX spike-in,
7 were loaded onto a MiSeq v3 flow cell, and sequenced using an Illumina MiSeq sequencer.
8 Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate
9 sequencing. De-multiplexing of reads was performed on instrument. Library preparation,
10 pooling, sequencing was performed at the Genome Research Core, Research Resources Center
11 (RRC), University of Illinois at Chicago (UIC).

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Author Contributions

E.I., A.G.E., R.T.A., W.R. R.A.H., M.H., S.C. and T.L. Preformed the work and contributed to data collection; E.I., A.G.E., P.F.B and S.L.H. designed the study and wrote the manuscript; B.K.L.S. P.F.B., A.G.E. and S.L.H. supervised the project. All authors discussed the results and commented on the manuscript.

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1 **Competing interests**

2 E.I., A.G.E., M.H., T.L., S.C., P.F.B., B.K.L.S., and S.L.H. are salaried employees of Sanaria Inc., the

3 developer and owner of PfSPZ Vaccine. In addition, S.L.H. and B.K.L.S. have a financial interest in

4 Sanaria Inc. No other authors have a competing interest.

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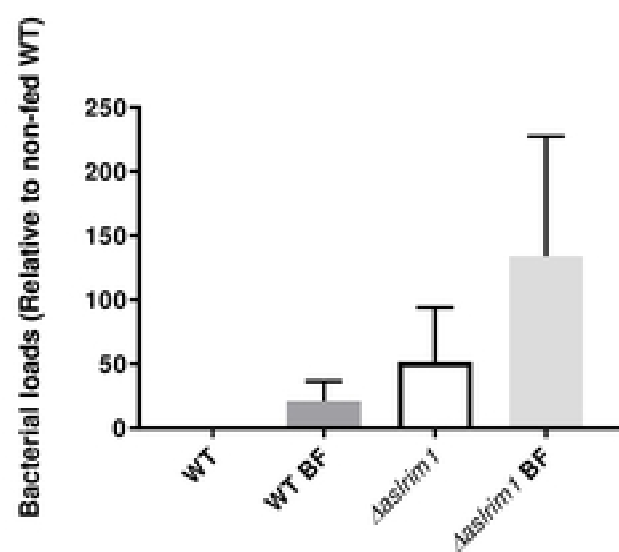
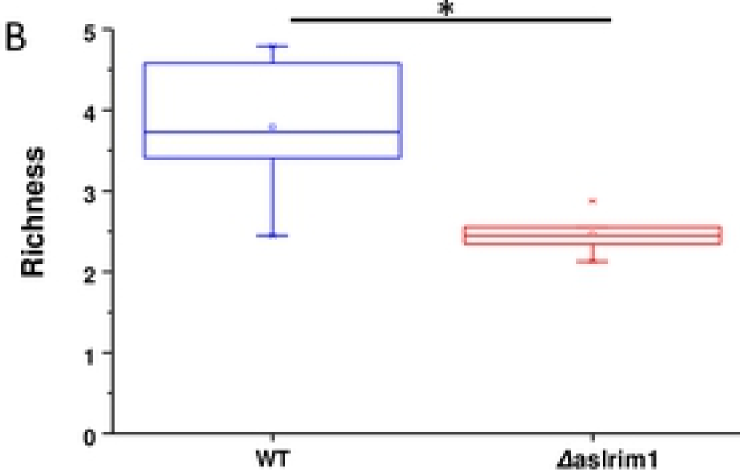
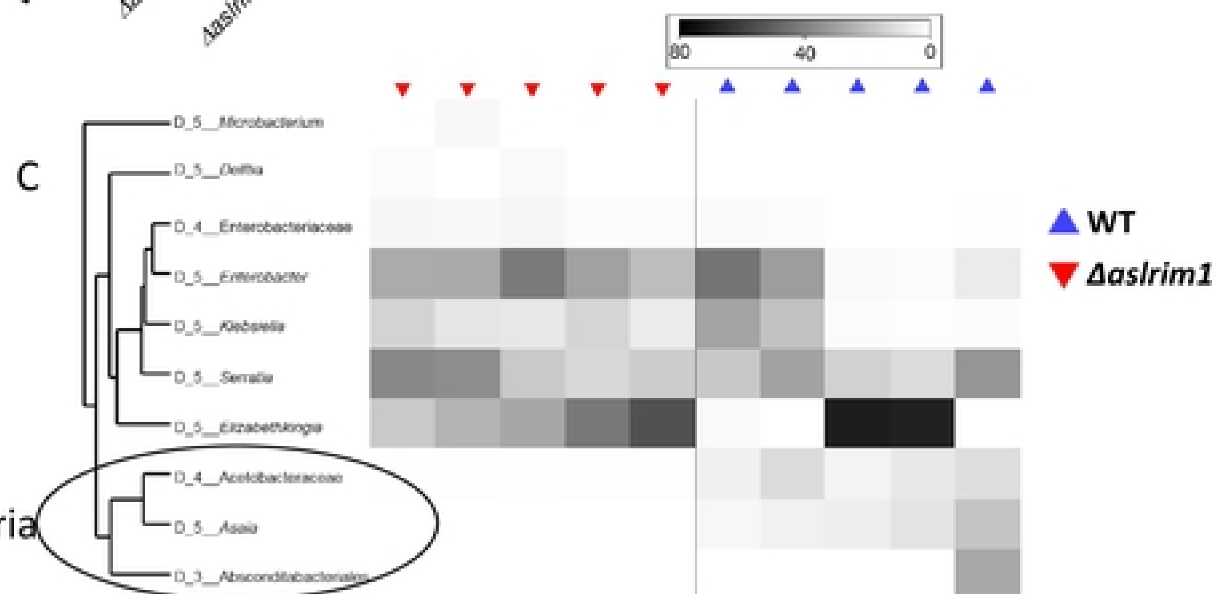
Fig 1







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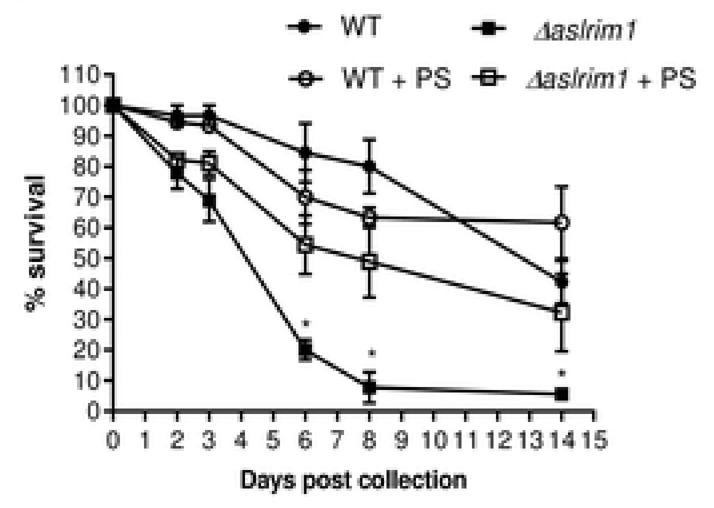

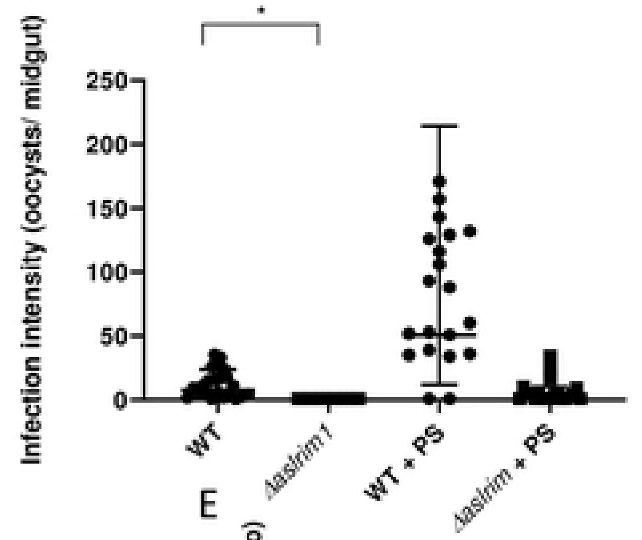

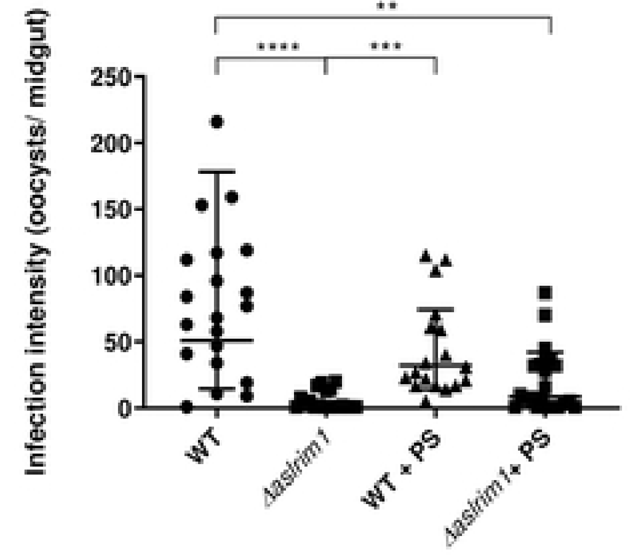

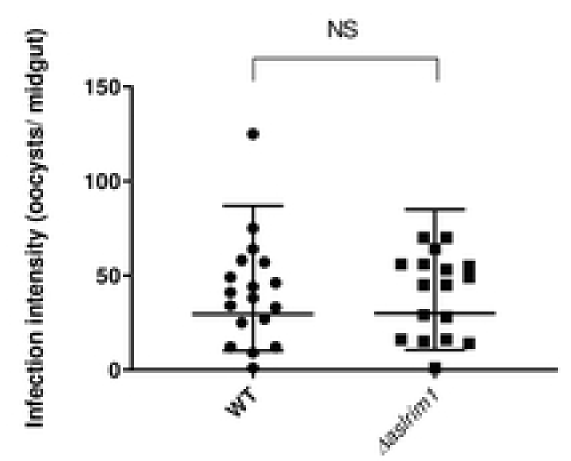

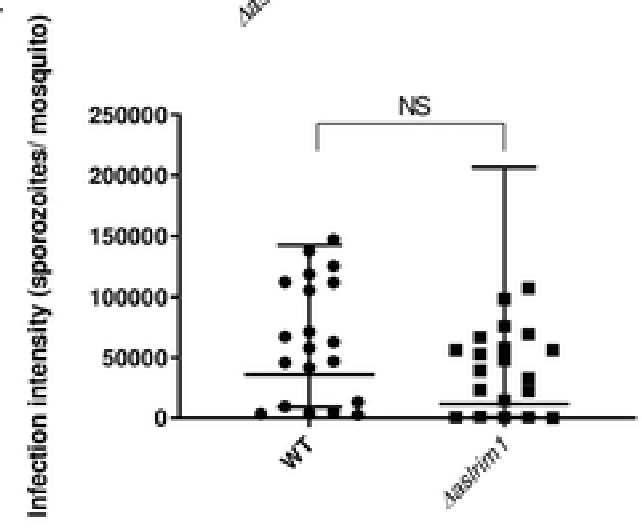

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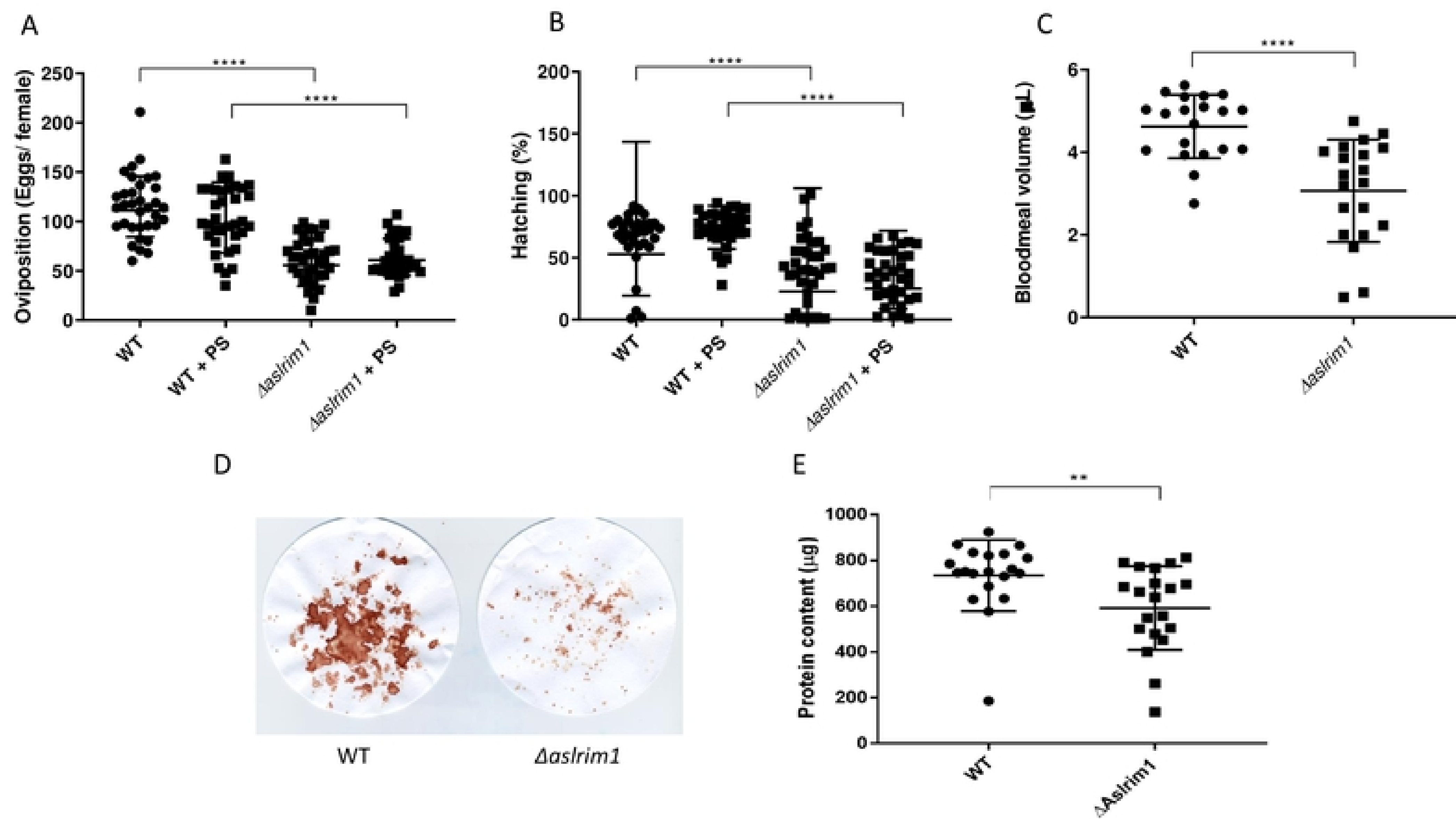

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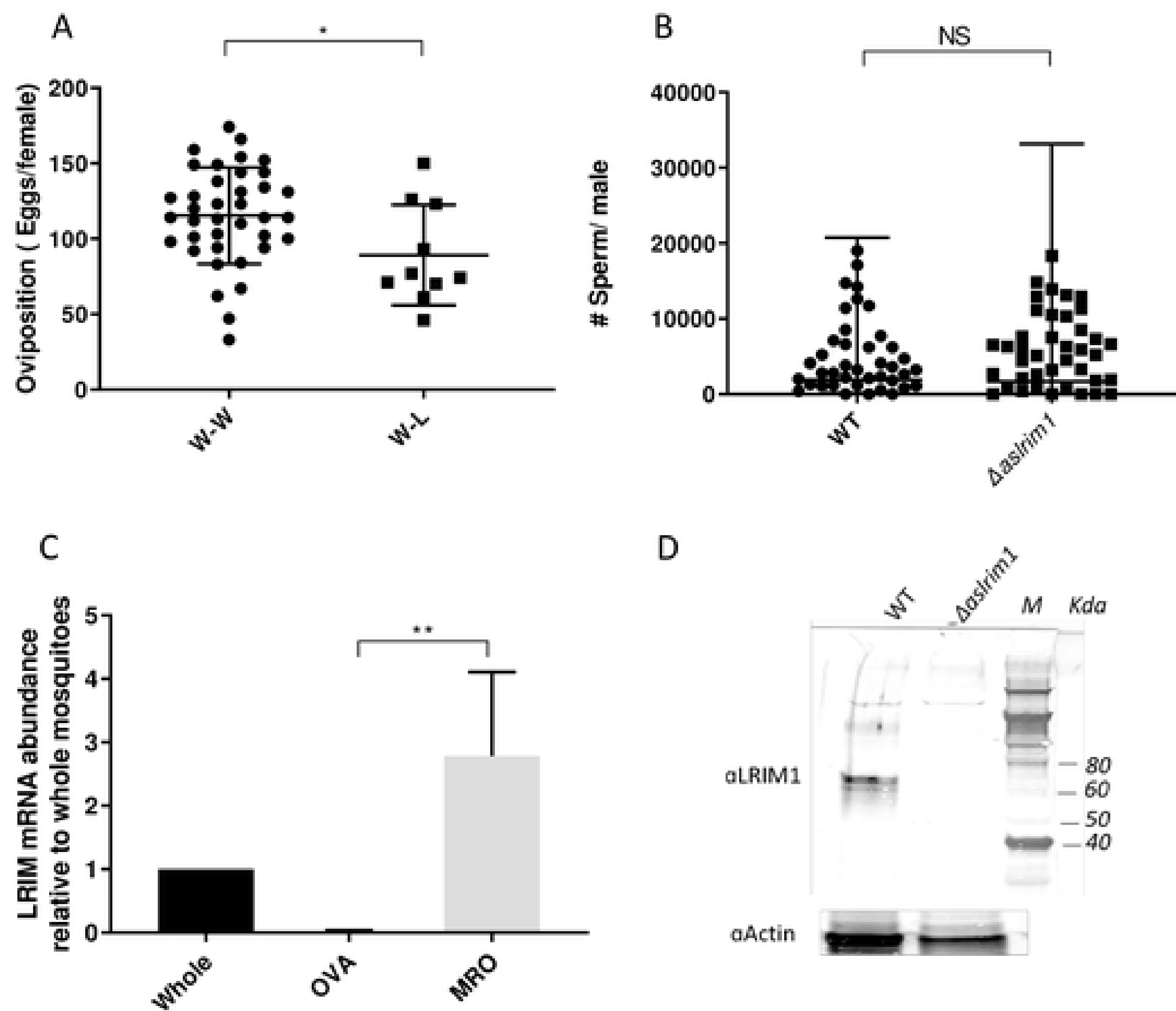
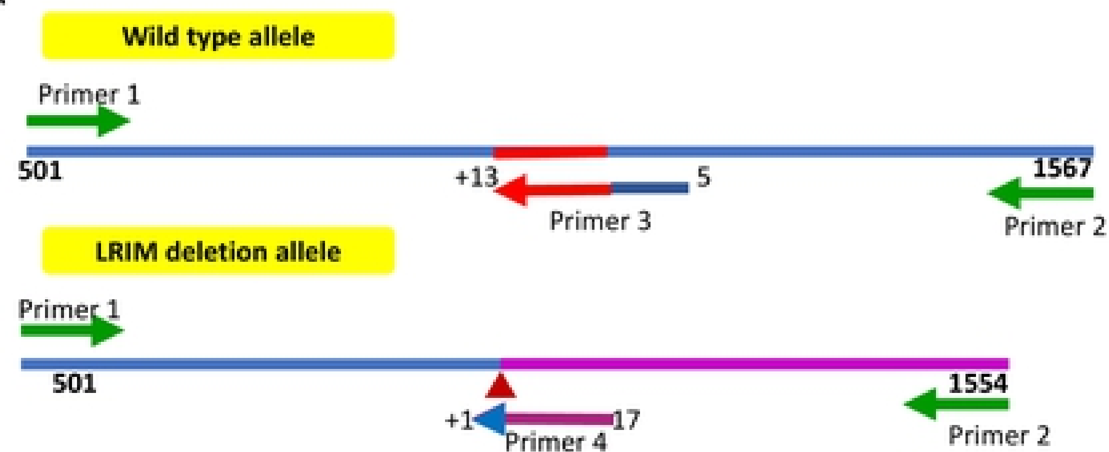
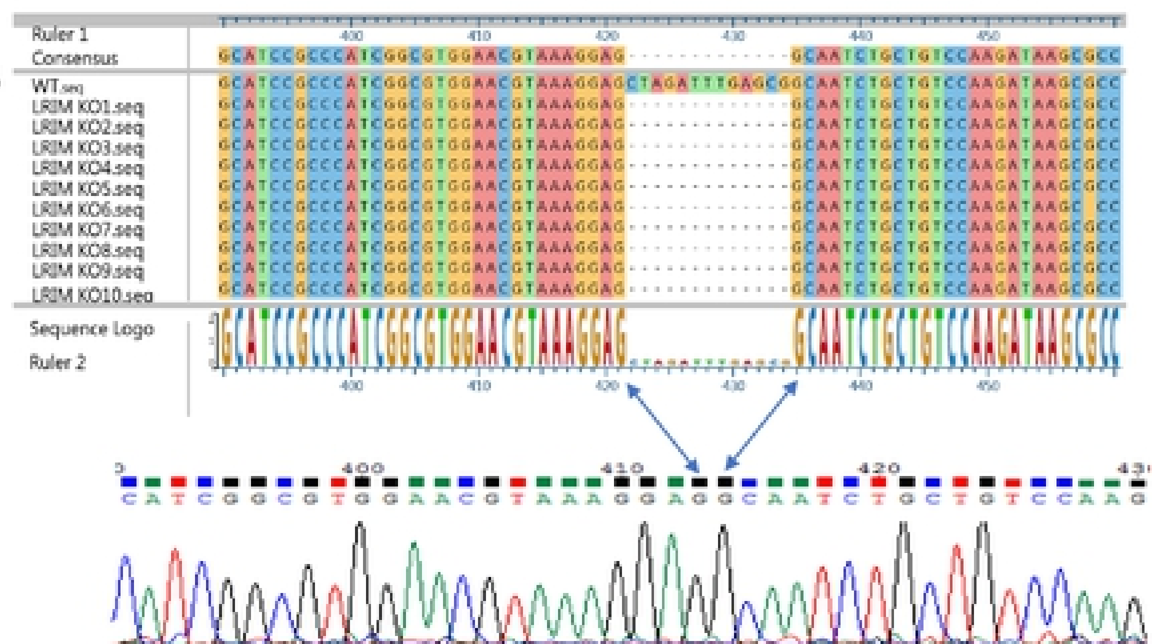


Fig S1





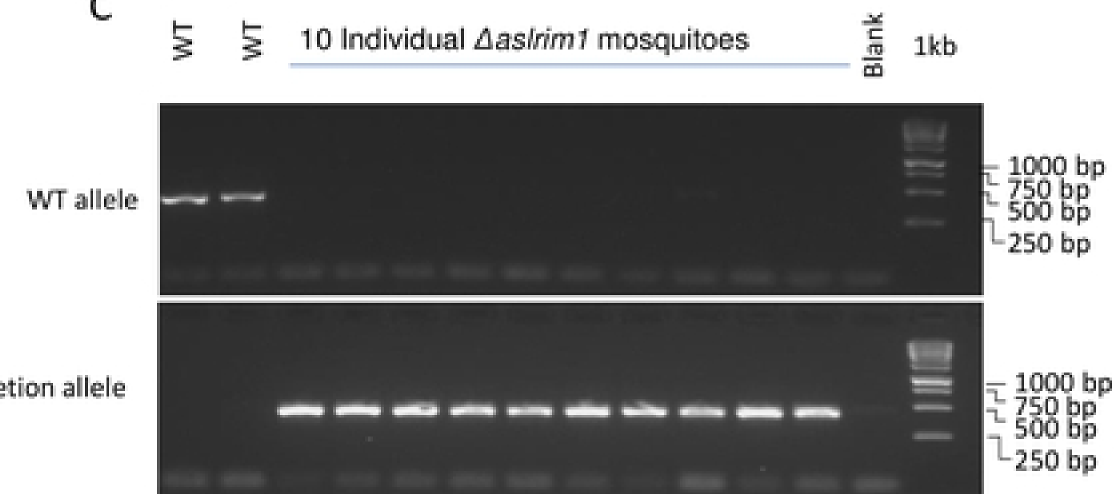
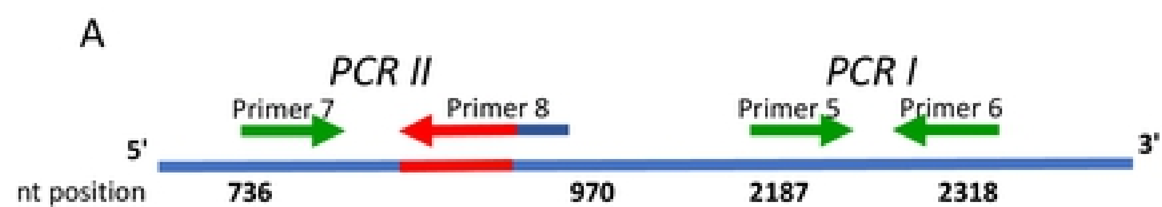
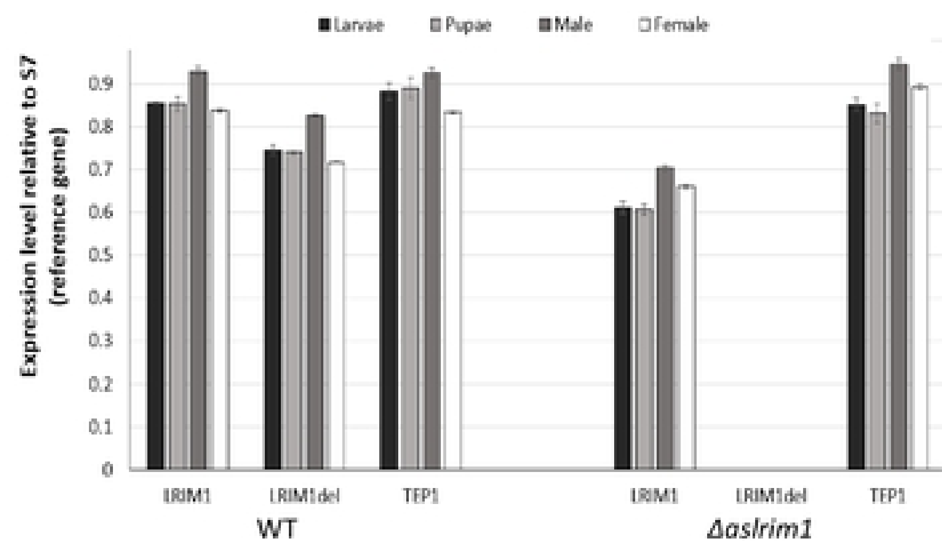


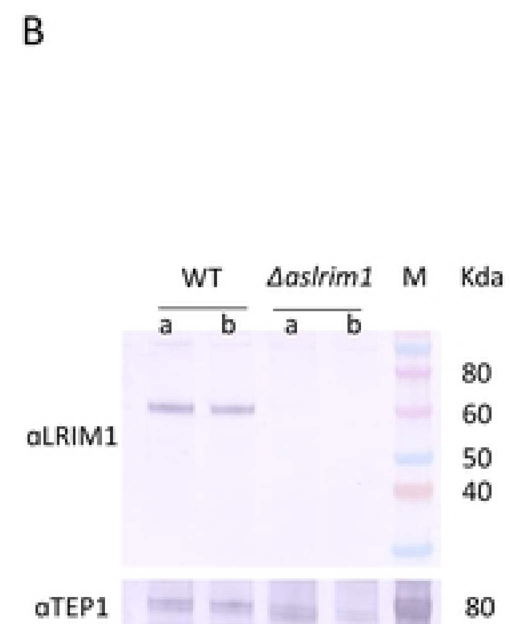
Fig S2

A





B



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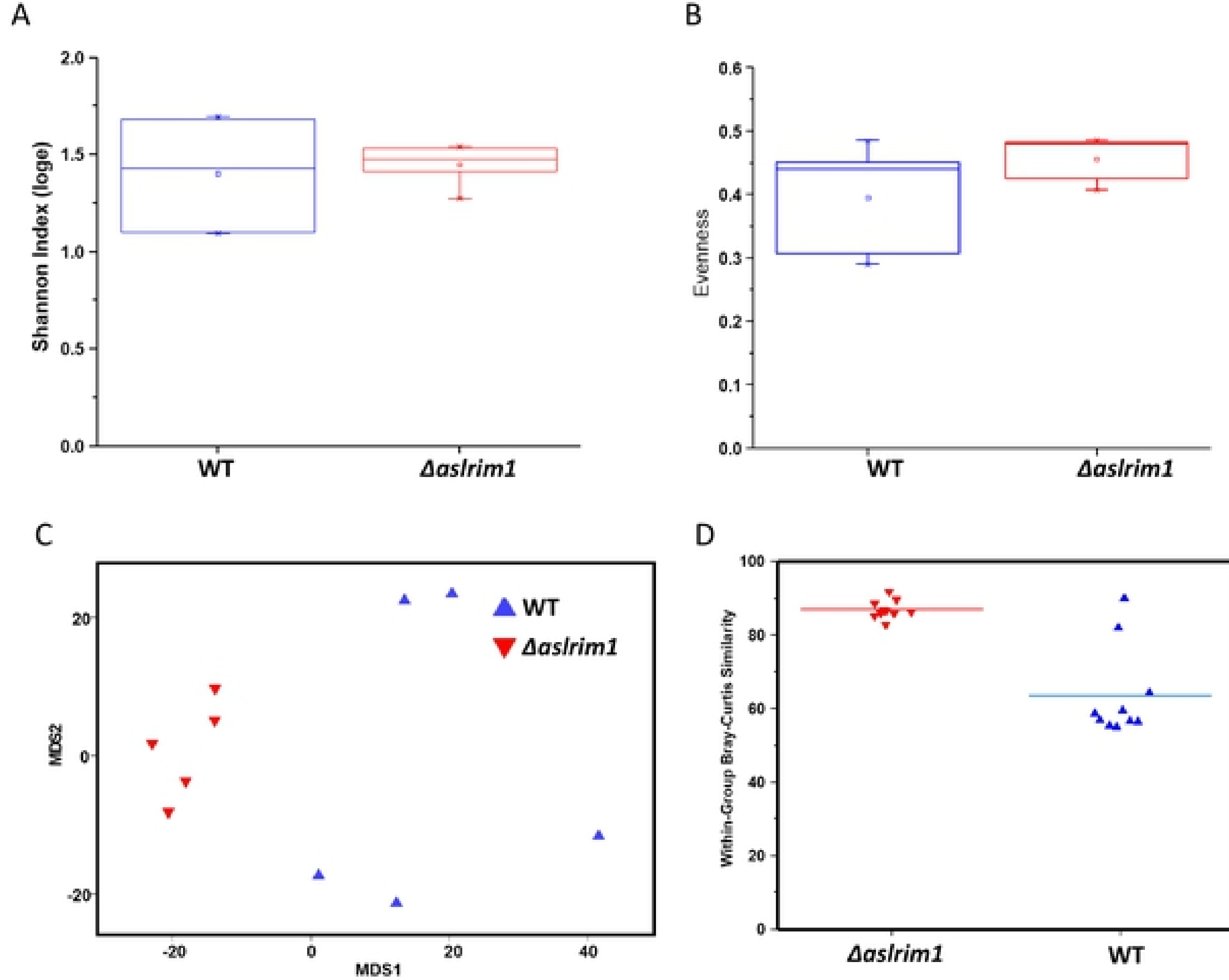

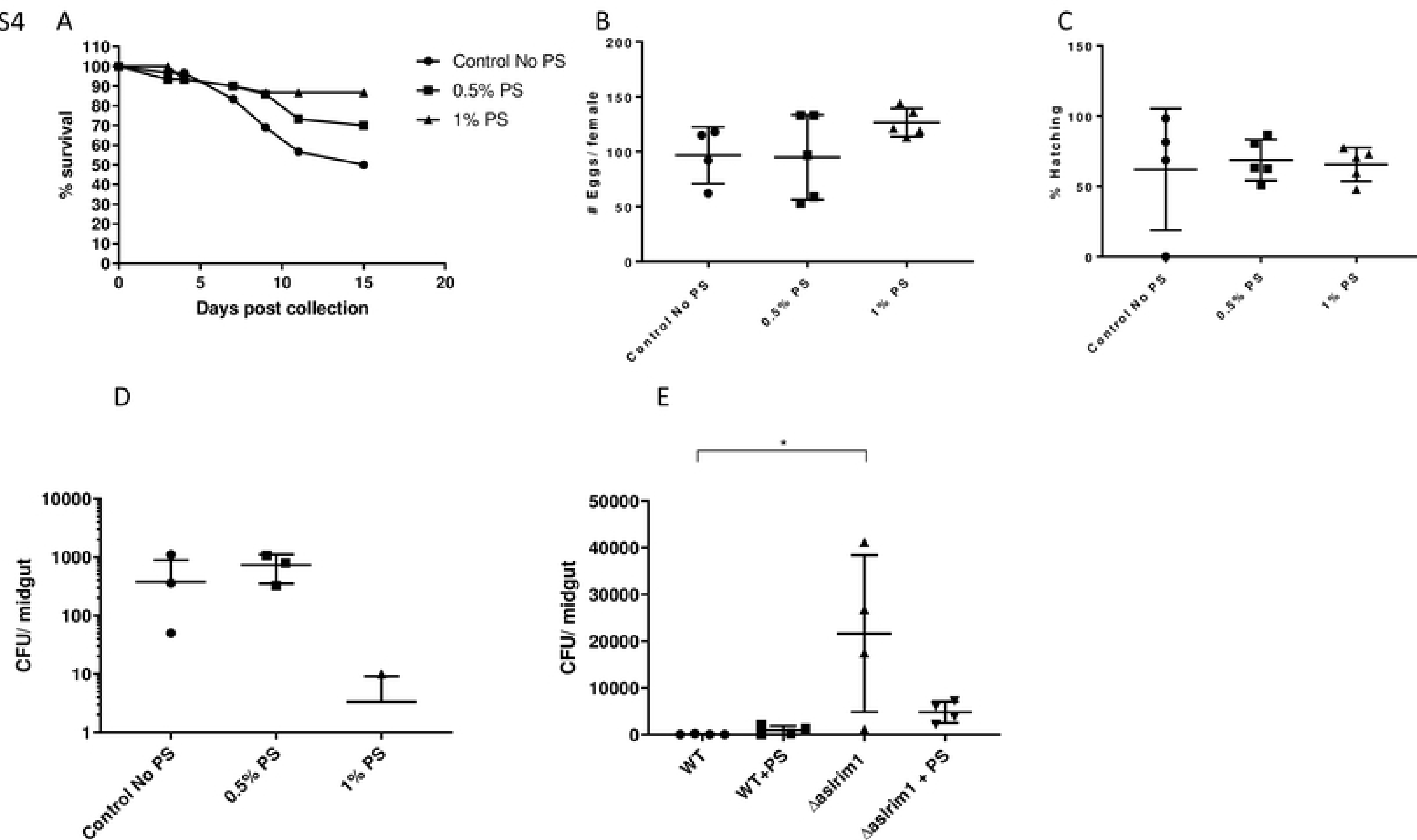

Fig S4
A
B
C
D
E
110
100
90
80
70
60
50
40
30
20
10
0
% survival
0
5
10
15
20
Days post collection
Control No PS
0.5% PS
1% PS
Eggs/ female
200
150
100
50
0
% Hatching
150
100
50
0
Control No PS
0.5% PS
1% PS
CFU/ midgut
10000
1000
100
10
1
50000
40000
30000
20000
10000
0
*
WT
WT+PS
Δaslrim1
Δaslrim1 + PS