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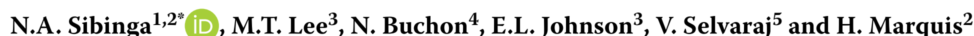
Do antimicrobial peptide levels alter performance of insect-based aquaculture feeds – a study using genetic models of insect immune activation

N.A. Sibinga[1,2*] , M.T. Lee[3], N. Buchon[4], E.L. Johnson[3], V. Selvaraj[5] and H. Marquis[2]

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Abstract

Insect meals are an increasingly common ingredient in pet food and animal feeds. Some studies have reported increased resistance to disease in animals fed insect-based diets, but the mechanisms of this effect have only rarely been investigated. We hypothesised that antimicrobial peptides (AMPs) naturally present in insects contribute to disease resistance. AMPs are part of the insect immune response, and as such their levels differ dramatically depending on the state of immune activation. In this study, transgenic fruit flies (*Drosophila melanogaster*) lines were used to generate insect-based feeds with high and low AMP levels. Juvenile rainbow trout (*Oncorhynchus mykiss*) were fed these insect diets and challenged by immersion with *Yersinia ruckeri* to assess changes in disease resistance. Survival rates of fish fed the high AMP insect diet were not significantly different from those fed the low AMP insect diet, though there were trends towards higher survival and lower *Y. ruckeri* load in the intestines of fish fed the high AMP diet. The fish intestinal microbiomes were studied in challenged and mock-challenged fish. In mock-challenged fish, no differences were apparent between the microbiomes of each diet group. In *Y. ruckeri* challenged fish, however, the intestinal microbial communities differed between fish fed the low and high AMP diets at each time point after infection. Overall, despite its limitations, this study offers a prospective view of how to leverage the genetic tools and experimental approaches present in the *Drosophila* community for research in commonly farmed insects (e.g. *Hermetia illucens* and *Tenebrio molitor*).

Keywords: *Drosophila*, infection challenge, *Yersinia ruckeri*, rainbow trout, Imd pathway

1. Introduction

Farmed insects have been highlighted as a promising alternative protein source to help meet the nutritional needs of a growing global population (Kim *et al.*, 2019). In addition to having excellent nutritional profiles, insect ingredients compare favourably to existing protein sources in terms of environmental impact; when compared to conventional livestock, insects have lower requirements for space, food, and water, as well as reduced emissions of greenhouse gases and ammonia (Van Huis *et al.*, 2013). Insect products have potential for human food applications (Doi *et al.*, 2021), but in recent years focus has primarily been on their use in animal feeds (Belghit *et al.*, 2018; Józefiak *et al.*, 2016). There has been particular interest in the use of insect ingredients in aquaculture feeds, where the levels of dietary protein are generally high and many aquatic species have limited tolerance for plant-derived protein ingredients (Hardy and Kaushik, 2021). Current evidence suggests that at moderate inclusion levels (up to ~30% of the diet), insect meals perform well in fish (Hua, 2021).

An intriguing finding of some studies of insect-based diets in aquaculture settings has been the observation of increased resistance to disease. These effects do not appear to be consistently repeatable but they have been reported across a variety of different labs, hosts, and pathogens – for a review, see Gasco *et al.*, (2021). The mechanisms by which any diet, let alone an insect-based diet, can improve disease resistance remain an open topic of study (Wu *et al.*, 2019; Koutsos *et al.*, 2022; Martin and Król, 2017). In aquaculture, multiple mechanisms appear to be supported by experimental data

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including effects related to modification of the microbiome (Infante-Villamil *et al.*, 2021; Legrand *et al.*, 2020), and regulation of immune effectors (Ali *et al.*, 2018; Lallès, 2019; Wan-Mohtar *et al.*, 2021). A diverse set of bioactive components of insect ingredients have been identified and tested in fish diets in purified form: chitin and chitosan (Riaz Rajoka *et al.*, 2020), lauric acid (Borrelli *et al.*, 2021), novel polysaccharides (Ali *et al.*, 2021), and antimicrobial peptides (Dai *et al.*, 2020) have all been shown to have the ability to improve fish resistance to disease. While these findings represent meaningful progress, there remains a gap in our understanding of how these components function in the context of non-purified insect ingredients (e.g. insect protein meals). In some notable cases, crude insect ingredients have shown protective effects against fish diseases (Ido *et al.*, 2015; Su *et al.*, 2017; Xiang *et al.*, 2020), but the scientific community currently lacks sufficient understanding to predict how and when crude insect ingredients will affect immune health.

Other authors have commented on the apparent inconsistency of results between studies of insect-based aquaculture feeds; this is usually attributed to variability between insect ingredients from different sources (Abdel-Tawwab *et al.*, 2020; Agbohessou *et al.*, 2021; Alves *et al.*, 2021; Bruni *et al.*, 2018). Differences in processing can cause meaningful differences in nutritional composition and digestibility for insect meals derived from the same species (Basto *et al.*, 2020; Oonincx and Finke, 2021). Furthermore, insect larvae show a great deal of plasticity in their nutrient profile based on rearing conditions and feed substrate – particularly with regard to the lipid fraction (Gasco *et al.*, 2021; Liland *et al.*, 2017; Oonincx *et al.*, 2015; Scala *et al.*, 2020). In addition to differences in nutritional composition, we hypothesised that variability in the levels of some or all of the bioactive components listed above could represent an important axis of variation between insect meals. We focused on antimicrobial peptides (AMPs), which have previously been shown to be differentially expressed between black soldier fly (*Hermetia illucens*) larvae reared on different feed substrates (Jin *et al.*, 2022; Vogel *et al.*, 2018).

AMPs are small cationic peptides (10-50 amino acids) that are a key part of the innate immune response in multicellular organisms. Insect AMPs have been studied in the context of immunology (Hanson and Lemaitre, 2020; Marra *et al.*, 2021), drug development (Manniello *et al.*, 2021; Mylonakis *et al.*, 2016), and, increasingly, as feed additives (Józefiak and Engberg, 2017). Several recent studies have examined the use of purified AMPs to improve fish resistance to disease (Chettri *et al.*, 2017; de Sousa *et al.*, 2021; Hu *et al.*, 2021; Rashidian *et al.*, 2021), though only a subset have used insect AMPs (Dai *et al.*, 2020; Lin *et al.*, 2015; Sibinga *et al.*, 2022). Some (though not all) of these studies have reported protective effects, but it is hard to extrapolate results from individual purified AMPs to the use of crude insect meals. For one thing, supplementation of feed with a single AMP is an imperfect model: insects produce many different AMPs and these are known to act synergistically (Hanson *et al.*, 2019; Marxer *et al.*, 2016). Another consideration is that it is difficult to isolate the effect of AMPs in many studies of insect-based diets, since those diets inevitably also contain chitin and lauric acid, among other potentially bioactive compounds. To elucidate the effects of AMPs in the context of insect ingredients, we therefore generated two insect meals differing in their level of AMPs.

To do this, we employed a genetic model of immune regulation using fruit flies (*Drosophila melanogaster*). In fruit flies, activation of the AMP response is stimulated by exposure to pathogen-associated molecular patterns via one of two major pathways: the immune deficiency (Imd) pathway responds primarily to gram-negative bacteria and the Toll pathway to gram-positive bacteria, yeast, and fungi (Buchon *et al.*, 2014) (Figure 1). These pathways are highly conserved, both between insects and vertebrates (Dushay and Eldon, 1998), and among commonly farmed insect species including mealworms (*Tenebrio molitor*) (Keshavarz *et al.*, 2020), house flies (*Musca domestica*) (Tang *et al.*, 2014), and silkworms (*Bombyx mori*) (Wang *et al.*, 2021). Use of the fruit fly model enabled us to manipulate the Imd pathway to generate fruit flies with either high or low expression of AMPs independent of their exposure to pathogens. Increased expression of AMP genes has been robustly linked to increased antimicrobial activity of insect extracts *in vitro* over three decades of research in *Drosophila* and other insects (Samakovlis *et. al*, 1990; Buchon *et. al*, 2009; Yi *et. al*, 2014). These fruit flies were processed into aquaculture diets and fed to fish for one month to evaluate the effect of AMPs on the fish microbiome. In a subsequent experiment, fish were fed the experimental diets and then subjected to a challenge with the gram-negative fish pathogen *Yersinia ruckeri*. In the absence of infection challenge, no differences between diet groups were observed. In challenged fish, there was not a statistically significant protective effect of the high AMP insect diet, though the trend was towards higher survival, lower pathogen load, and a more stable microbiome in these fish.

2. Materials and methods

Drosophila care and processing

Two lines of transgenic fruit flies (*D. melanogaster*) were reared in plastic vials on standard fly medium (sucrose, cornmeal, yeast, agar) at room temperature (~22 °C). One line, *Rel*$^-$, was selected to have low expression of antimicrobial peptides (AMP). *Rel*$^-$ is an extensively-studied immune-deficient line that lacks the gene encoding

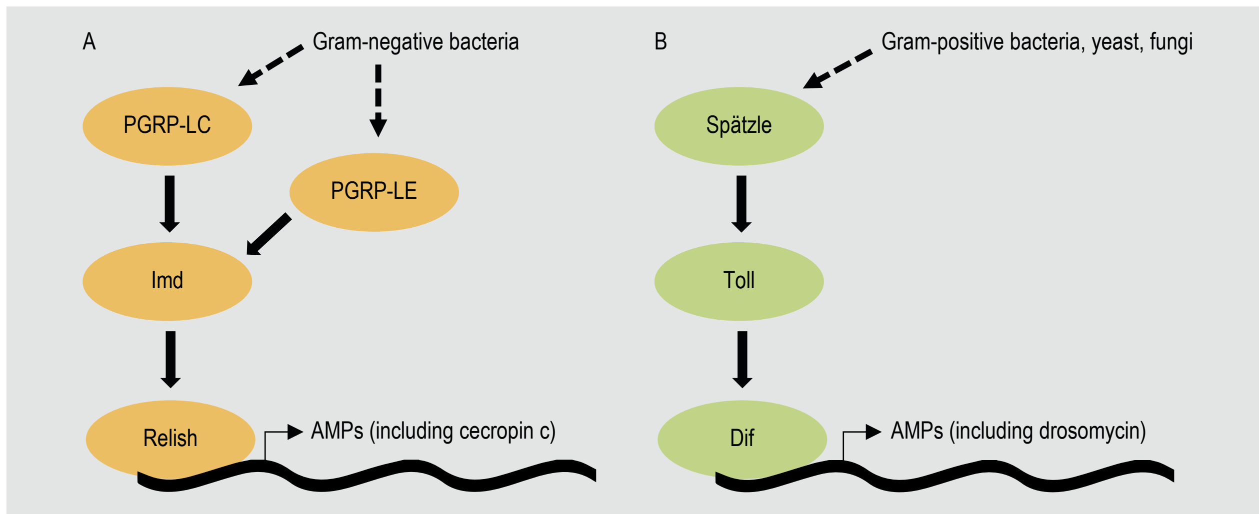

Figure 1. Summary of the major insect immune pathways. Antimicrobial peptides (AMPs) are a major component of the insect immune response and are primarily induced by one of two key pathways. (A) In the Imd pathway, peptidoglycan recognition proteins (PRGP) are activated by gram-negative bacteria and in turn activate Imd. This leads to a signal cascade that culminates with the nuclear factor kappaB (NF-κB) transcription factor Relish stimulating expression of AMPs. (B) The Toll pathway responds to gram-positive bacteria, yeast, and fungi via proteolytic release of the cytokine Spätzle, which binds to the Toll receptor. The Toll signal cascade results in translocation of Dif (another NF-κB transcription factor) to the nucleus and transcription of AMPs. Individual AMPs may be activated by either or both pathways, but the overall suite of induced AMPs is markedly different depending on the pathogenic stimulus.

the transcription factor Relish – essential for induction of the humoral immune response via the Imd pathway (Hedengren *et al.*, 1999). The second line, *HS_imd*, was selected to have high expression of AMPs. *HS_imd* utilises a heat-shock/GAL4 promoter system (hs-Gal4 > UAS-Imd). Due to technical limitations, heat shocking the large numbers of flies required for this study proved impractical. However, both the initial experiments using this line (Georgel *et al.*, 2001) and our own preliminary experiments (Supplementary Figure S1) demonstrate that this is a leaky promoter system that results in constitutive expression of AMPs regulated by the Imd pathway even under stable room temperature conditions. Adult flies from each line were collected once per week over the course of three months directly into liquid nitrogen and stored at -80 °C. At the end of the collection period, flies were lyophilised (Izutsu, 2014) and ground into meal using an electric burr grinder.

RNA extraction from *Drosophila*, reverse transcription, and qPCR

Preliminary experiments were conducted to confirm upregulation of AMPs in the high AMP *Drosophila* line. Three groups of triplicate vials of newly emerged *HS_imd* flies were maintained at room temperature. After one week, one of the groups was heat-shocked by submerging the vials in a 37 °C water bath for 1 h. The following day, this group and one of the naïve groups were heat shocked. On the third day, 10 flies from each vial of each of the three groups (naïve, heat-shocked 1×, heat-shocked 2×), as well as an additional group comprised of age-matched Rel$^-$ flies, were collected for extraction of RNA using Trizol reagent (Invitrogen, Carlsbad, CA, USA).

Flies were collected directly into Trizol reagent (Invitrogen) on ice and homogenised for 30 s on a bead beater with 5-10 1.3-mm chrome steel beads. Homogenates were incubated at 4 °C overnight before RNA extraction. RNA was extracted as per manufacturer instructions with the following modification: prior to precipitation of RNA, the aqueous phase containing nucleic acids was re-extracted with Trizol reagent once. RNA and DNA levels were quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) to verify extract quality and purity. cDNA libraries were then generated for each sample using the SuperScript IV First-Strand Synthesis System (Invitrogen). Multiplex quantitative polymerase chain reaction (qPCR) was performed on a 7500 real-time PCR instrument (Applied Biosystems, Bedford, MA, USA) and used to compare expression levels of representative AMP genes (see Supplementary Table S1 for primer sequences). The *Drosophila cecropin c* gene is regulated by the Imd pathway, while *drosomycin* is regulated by the Toll pathway. Expression of both genes was normalised by comparison to the *Rpl32* housekeeping gene (Ponton *et al.*, 2011) and the expression levels in *Rel*$^-$ flies using the double delta Ct method (Livak and Schmittgen, 2001). To confirm the stability of AMP gene expression over multiple generations, 10 flies were taken from each of several collection dates

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spanning the entirety of the three-month collection period and analysed as described above.

Diet formulation and analysis

Two diets were used for this experiment. These diets incorporated insect meal generated from either low AMP (Rel$^-$) or high AMP (HS_imd) *Drosophila* lines, and were formulated to meet the published nutritional requirements of juvenile rainbow trout (National Research Council, 2011). Formulation relied on published values to estimate the nutritional composition of *Drosophila* adults. Proximate analysis of *Drosophila* insect meal and all diets was performed by Exact Scientific Services (Ferndale, WA, USA) at the end of the study (Table 1). Due to a lower than anticipated protein content in the transgenic *Drosophila*, these diets were below the published protein requirement of 39.5% for rainbow trout of this size (Table 2). During production and storage of all diets, care was taken to keep ingredients cold to minimise potential degradation of AMPs. Dry ingredients were combined on ice, oils were added, then just enough cold distilled water was added to form a dough. This dough was immediately extruded through a pre-chilled single screw extruder directly into liquid nitrogen and resulting pellets were lyophilised. All diets were stored at -20 °C until use.

Fish care and handling

All animals were cared for following the 'Guide for the Care and Use of Laboratory Animals' and American Association of Laboratory Animal Science Position Statements; the Cornell University Institutional Care and Use Committee (IACUC) reviewed and approved all experimental protocols. Rainbow trout (*Oncorhynchus mykiss*) fry from the New York State fish hatchery at Bath, NY, USA, were acclimated to the Cornell aquatic facility in a single 700-litre fiberglass tank under flowthrough conditions for three weeks and fed BioClarks Fry 1.2-mm pellets (Bio-Oregon, Longview, WA, USA). In the third week of acclimation water temperature was incrementally increased from 12±1 to 15±1 °C. After acclimation, fish were assigned to experimental treatments in the first trial or retained in a separate tank on BioClarks Fry pellets for use in the second trial.

Yersinia ruckeri challenge and sampling

The challenge and mock-challenge conditions in this study are identical to those described in another study that was conducted concurrently (Sibinga *et al.*, 2022). Briefly, challenged fish were immersed for 1 h in a suspension of *Y. ruckeri* strain CSF007-82 at a concentration of ~8×10^8 cfu/ml in 10-litre flowthrough tanks with supplemental

Table 1. Composition of diets (g/kg).

Ingredients	Company	g/kg
Insect meal		200
Menhaden fishmeal	Omega Protein® Special Select Menhaden, Reedville, VA, USA	150
Wheat gluten	Betta Foods, Inc., Parrottsville, TN, USA	50
Wheat flour	King Arthur Flour Company, Inc., Norwich, VT, USA	311
Cod liver oil	Piping Rock Health Products, LLC, Ronkonkoma, NY, USA	110
Soy protein concentrate	The Sausage Maker, Inc., Buffalo, NY, USA	134
Soybean oil	C & S Wholesale Grocers, Inc., Worchester, MA, USA	34
Mineral premix	Florida Aqua Farms, Inc., Dade City, FL, USA	1
Vitamin premix	Florida Aqua Farms, Inc., Dade City, FL, USA	10

Table 2. Proximate composition of experimental insect meals and diets (g/kg).[1]

	High AMP insect meal	Low AMP insect meal	High AMP insect diet	Low AMP insect diet
Dry matter	946	937	895	889
Lipid	166	118	n.t.	185
Protein	540	540	342	372
Carbohydrates	198	234	n.t.	280
Ash	42	46	53	52

[1] AMP = antimicrobial peptides; n.t. = not tested; i.e. insufficient sample for analysis.

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aeration. Mock-challenged fish were immersed in an equivalent volume of sterile tryptic soy broth (TSB). After 1 h, water flow was restored and after 24 h fish were returned to their original 700-litre tanks and maintained on the experimental diets for one month. Challenged fish displayed decreased appetite between days 3 and 14 post-infection; accordingly, daily feeding was reduced for this period from 3% of total estimated bodyweight to 1% of total estimated bodyweight. Sampling occurred at the following time points: immediately prior to challenge/mock-challenge (day 0), and then at 3-, 8-, and 30-days post-challenge. Sampled fish were euthanised via an overdose of buffered MS-222 (Syndel, Ferndale, WA, USA) prior to aseptic removal of the whole intestine posterior of the pyloric caeca. Intestine samples were flash frozen and stored at -80 °C.

In the first trial, fish (average body weight = 2.5 g) were assigned to the low AMP or high AMP insect diet and then, three days after introduction of the experimental diets, either challenged with *Y. ruckeri* (50 fish per diet) or mock-challenged with TSB (28 fish per diet). Challenged fish in this trial sustained very high mortality, resulting in too few samples for informative analysis of the complete time course. Accordingly, the challenge treatment (but not the mock-challenge) was repeated in a second trial with larger fish from the same cohort (average body weight = 4.0 g) and larger sample sizes. Fish were culled at 3-, 8-, and 30-days post-infection and sampled were censored from the data at the appropriate time points for survival analysis.

DNA extraction from fish intestines

Extraction of DNA from trout intestines was performed as previously described (Sibinga and Marquis, 2021). Whole intestines (and contents) were weighed prior to addition of DNA extraction buffer (200 mM NaCl, 200 mM Tris-HCl pH 7.5, 20 mM EDTA, 5% SDS). Subsequently, samples were homogenised on a Mini-Beadbeater (BioSpec Products, Inc. Bartlesville, OK, USA) with 1.3-mm chrome steel beads. A secondary homogenisation step with 0.1-mm zirconia/silica beads was performed on a subsample of the initial homogenate corresponding to 20 mg of intestinal tissue and contents. DNA was extracted from the secondary homogenate via phenol:chloroform extraction and isopropanol precipitation.

qPCR detection and quantification of the *Yersinia ruckeri* 16S rRNA gene

To determine the pathogen load of challenged fish over time, we used a highly sensitive qPCR assay to detect the *Y. ruckeri* 16S rRNA gene in intestinal DNA extracts of infected fish from the second trial. Primer and probe design followed the work of Ghosh and colleagues (Ghosh *et al.*, 2018) (Supplementary Table S1), and targeted the V2-V3 region of the 16S gene. Triplicate 10 µl reactions were performed in 96-well plates using the following reaction mix: 2X PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA, USA), forward and reverse primers (400 nM each), TaqMan probe (100 nM), DNA template (1 µl), and ultrapure water and plates were run on a 7500 real-time PCR instrument (Applied Biosystems). Samples with amplification of the 16S gene in at least two of three replicate qPCR wells were deemed positive for *Y. ruckeri*. A standard curve generated using genomic DNA from a pure culture of *Y. ruckeri* was used to estimate bacterial loads as previously described (Sibinga and Marquis, 2021). The limit of quantitation (LOQ) was determined using a published R script (Merkes *et al.*, 2019); the LOQ is the lowest threshold count (Ct) value for which the coefficient of variance – i.e. the standard deviation expressed as a percentage of the mean (Forootan *et al.*, 2017) – is less than 35% based on the results of the standard curve.

PCR amplification of V4 region of 16S rRNA gene for DNA sequencing

In preparation for sequencing, DNA extracts were diluted 1:10 in ultrapure water and amplified via PCR using the 515F and GoLay-barcoded 806R universal 16S primers (Takara Bio, Mountain View, CA, USA), which target the V4 region of the 16S gene (Caporaso *et al.*, 2011). Amplification (3 min. 94 °C; 30 cycles of 45 s 94 °C, 60 s 50 °C, 90 s 72 °C; 10 min. 72 °C) was performed in duplicate, after which duplicates were pooled. The Mag-Bind® RxnPure Plus kit (Omega Bio-tek, Inc., Norcross, GA, USA) was used to purify amplicons before 150 ng of each amplicon sample were pooled for sequencing. Paired-end sequencing (2×250 bp) was performed by the Genomic Facility at Cornell Institute of Biotechnology (Ithaca, NY, USA) on an Illumina MiSeq sequencer (San Diego, CA, USA).

Analysis of 16S rRNA gene sequences

The QIIME 2 (v 2020.2) pipeline was used for analysis and organisation of raw sequence data (Caporaso *et al.*, 2010). Analysis of sequence quality and assignment of amplicon sequence variants (ASVs) was performed with the Divisive Amplicon Denoising Algorithm 2 (DADA2) (Callahan *et al.*, 2016); samples with greater than 50% chimeric reads were excluded from further analysis. ASVs were mapped to the Greengenes 16S rRNA database for assignment of taxonomic identities (McDonald *et al.*, 2012). ASV_3428 was identified as *Y. ruckeri* by additional methods described in a previous study (Sibinga *et al.*, 2022): briefly, ASV_3428 relative abundance was strongly correlated with qPCR detection and quantification data and ASV_3428 displays perfect identity to the published 16S gene sequence of the *Y. ruckeri* strain used in this study. The diversity plugin

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for QIIME2 was used to calculate Bray-Curtis distances between all samples for analysis of beta-diversity (Bolyen *et al.*, 2019). Rstudio (v 1.0.136) was used for subsequent analysis of QIIME2 output data with the following packages: phyloseq (McMurdie and Holmes, 2013), microbiome (Lahti and Shetty, 2017), tidyverse (Wickham *et al.*, 2019), EnhancedVolcano (Blighe *et al.*, 2021), and ggplot2 (Wickham, 2016).

Statistical analysis

Gene expression data was analysed using Quantstudio Design and Analysis software (v 2.6.0; Thermo Fisher Scientific). Fish survival data was analysed using a Kaplan-Meier function and Gehan-Breslow-Wilcoxon test in JMP Pro 14. *Y. ruckeri* detection data was analysed with Fisher's exact test; quantification data was analysed by analysis of variance (ANOVA) including only samples above the LOQ. Sequence data was analysed using Rstudio (Posit Software, Boston, MA, USA) and QIIME 2. Shannon index values were analysed by ANOVA with post-hoc pairwise Tukey tests. Bray-Curtis distances for group and individual pairwise comparisons were evaluated using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations and Benjamini/Hochberg FDR *P*-value correction. DESeq2 (Love *et al.*, 2014) was used to identify significant differences in the abundance of individual ASVs between groups.

3. Results

Expression of AMPs was upregulated in the high AMP *Drosophila* line

Preliminary experiments demonstrated that untreated flies from the high AMP *Drosophila* line (*HS_imd*) had approximately 24-fold upregulated expression of *cecropin c* compared to the low AMP line (*Rel-*) (Figure 2). Heat-shocking the high AMP line induced an even stronger response, with 105-fold upregulation of *cecropin c* (Supplementary Figure S1), though due to technical limitations we were not able to use heat-shocked flies for production of insect meal. *Drosomycin*, an AMP regulated by the Toll pathway, was upregulated 2.7-fold in heat shocked high AMP flies but was not upregulated in untreated flies (Supplementary Figure S1). Subsampling of high AMP flies from different harvest dates revealed that the observed gene expression levels were stable across generations (Supplementary Figure S2).

The high AMP diet did not significantly protect against *Yersinia ruckeri* challenge

A first infection challenge trial was performed concurrently with the mock-challenge trial, however survival rate in this experiment was very low in both the high AMP (1 fish, 6%) and low AMP (2 fish, 7%) diet groups (data not shown). This left insufficient samples for analysis of the final time point; therefore this trial was excluded from further analysis. The challenge experiment was repeated at the conclusion of the first trial using larger sample sizes of trout from the same cohort. These fish were one month older and therefore also larger than the fish in the first trial. In the second challenge experiment, the survival rates were 48 and 27% for fish fed the high AMP and low AMP diets, respectively (Figure 3). The trend towards higher survival rate of fish fed the high AMP diet in the second trial was not statistically significant (*P*=0.26).

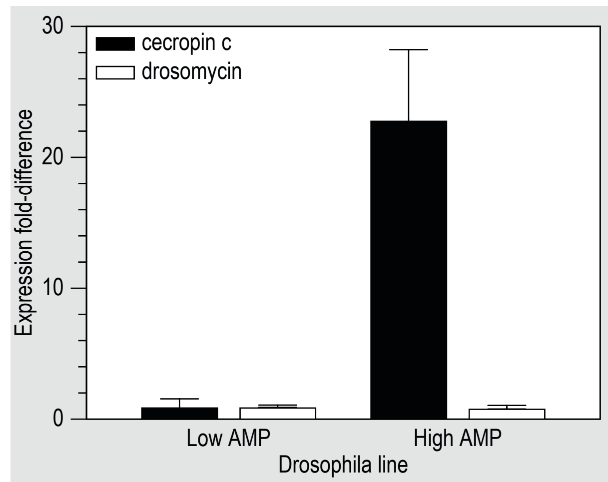

Figure 2. Difference in expression levels of two antimicrobial peptide (AMP) genes between the low and high AMP fly lines. We used transgenic *Drosophila* lines differing in the Imd pathway, a major regulator of AMP expression. The low AMP line has a non-functional Imd pathway, while the high AMP line expresses Imd constitutively. We collected adult flies from large colonies of each line for a period of three months. Shown above are mean gene expression levels of flies from each line over the collection period for *cecropin c*, a representative AMP regulated by the Imd pathway. For comparison, a representative AMP under control of the Toll pathway, *drosomycin*, is also shown. Expression levels of *cecropin c*, but not *drosomycin*, were many times higher in the high AMP line throughout the collection period.

The high AMP diet did not significantly reduce *Yersinia ruckeri* prevalence or abundance

To assess whether AMP levels in the insect diets might help to protect fish from colonisation by *Y. ruckeri*, we used qPCR to detect the *Y. ruckeri* 16S rRNA gene in intestinal DNA extracts. All fish from both diet groups were positive for *Y. ruckeri* at the day 3 and day 8 time points (Table 3). At day 30, 100% (6/6) of surviving fish from the low AMP insect diet were positive while 90% (9/10) fish from the high AMP diet were positive. We next used the qPCR results to estimate the *Y. ruckeri* load in intestines of challenged fish (Figure 4). The LOQ in this study was 13.86 gene copies,

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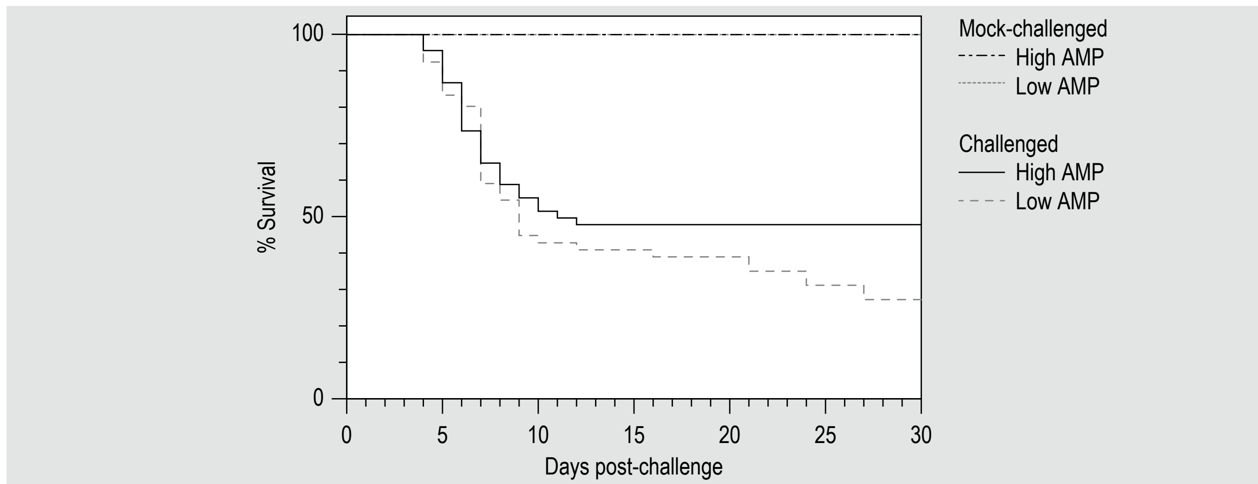

Figure 3. Kaplan-Meier survival curve for fish fed either the high antimicrobial peptide (AMP) insect diet or the low AMP insect diet following immersion challenge with *Yersinia ruckeri*. The difference in survival between the diet groups was not statistically significant (*P*=0.26). No mortality was observed in mock-challenged fish fed the experimental diets.

Table 3. Detection of the *Yersinia ruckeri* 16S gene by qPCR in intestinal DNA extracts.[1]

Diet group	Time post-infection		
	3 days	8 days	30 days
Low-AMP insect diet	8/8 (100%)[2]	8/8 (100%)	6/6 (100%)
High-AMP insect diet	8/8 (100%)	8/8 (100%)	9/10 (90%)

[1] AMP = antimicrobial peptide.
[2] Fish positive for *Y. ruckeri* by qPCR over total number of sampled fish with % in parentheses. Comparisons between diet groups were performed separately for each time point using Fisher's exact test. No statistically significant differences were found between diet groups for any time point.

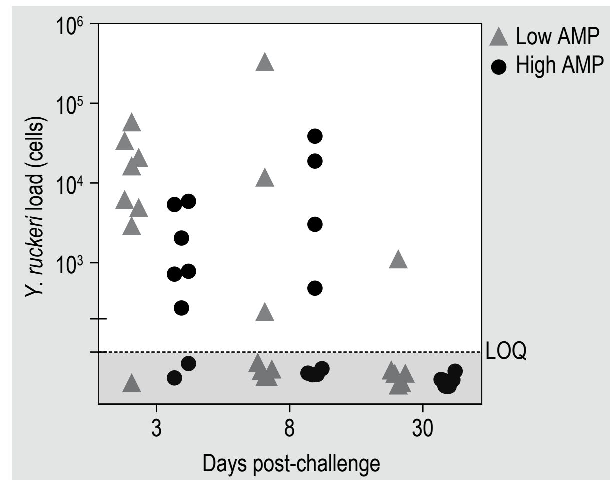

Figure 4. Estimated *Yersinia ruckeri* burden per 20 mg of intestinal tissue. The number of *Y. ruckeri* cells in each intestinal sample was estimated by qPCR using a standard curve. No estimate was made for samples below the limit of quantitation (LOQ) estimated at 198 cells – samples that were positive for *Y. ruckeri* but below the LOQ are displayed in the gray box. An ANOVA test of all samples above the LOQ revealed no significant effect of diet on *Y. ruckeri* load (*P*=0.249). AMP = antimicrobial peptide.

which corresponds to ~198 bacteria per 20 mg sample of intestine; no estimate of *Y. ruckeri* load was generated for samples with detectable loads below this threshold. At day 3, for samples above the LOQ, the median load of *Y. ruckeri* was ~1.1×10^4 cells/20 mg intestine in the low AMP insect diet compared to ~7.9×10^2 cells/20 mg intestine in the high AMP insect diet (Figure 4). This suggests potentially increased colonisation of the intestine during the early stage of infection in fish fed the low AMP diet, though this difference was again not statistically significant (*P*=0.25). This pattern was less pronounced at day 8 and day 30, though the highest observed *Y. ruckeri* loads for each of these time points were also from fish fed the low AMP diet.

Fish intestinal microbiomes in mock-challenged fish fed either insect diet: no changes in beta diversity

To understand how AMPs impacted commensal bacteria in the absence of infection, we compared the microbiomes of mock-challenged fish fed either the low AMP or high AMP insect diet (Figure 5A). The internal (alpha) diversity of the microbiomes did not change over the course of the experiment for fish fed the high AMP diet (Figure 5B). In fish fed the low AMP diet, however, there was a significant increase in alpha diversity between the day 0 time point (corresponding to 3 days after introduction of the experimental diet) and all subsequent sampling dates (Figure 5B). Multivariate comparison of between-sample (beta) diversity using PERMANOVA showed no significant effect of diet or timepoint on the microbiomes of uninfected

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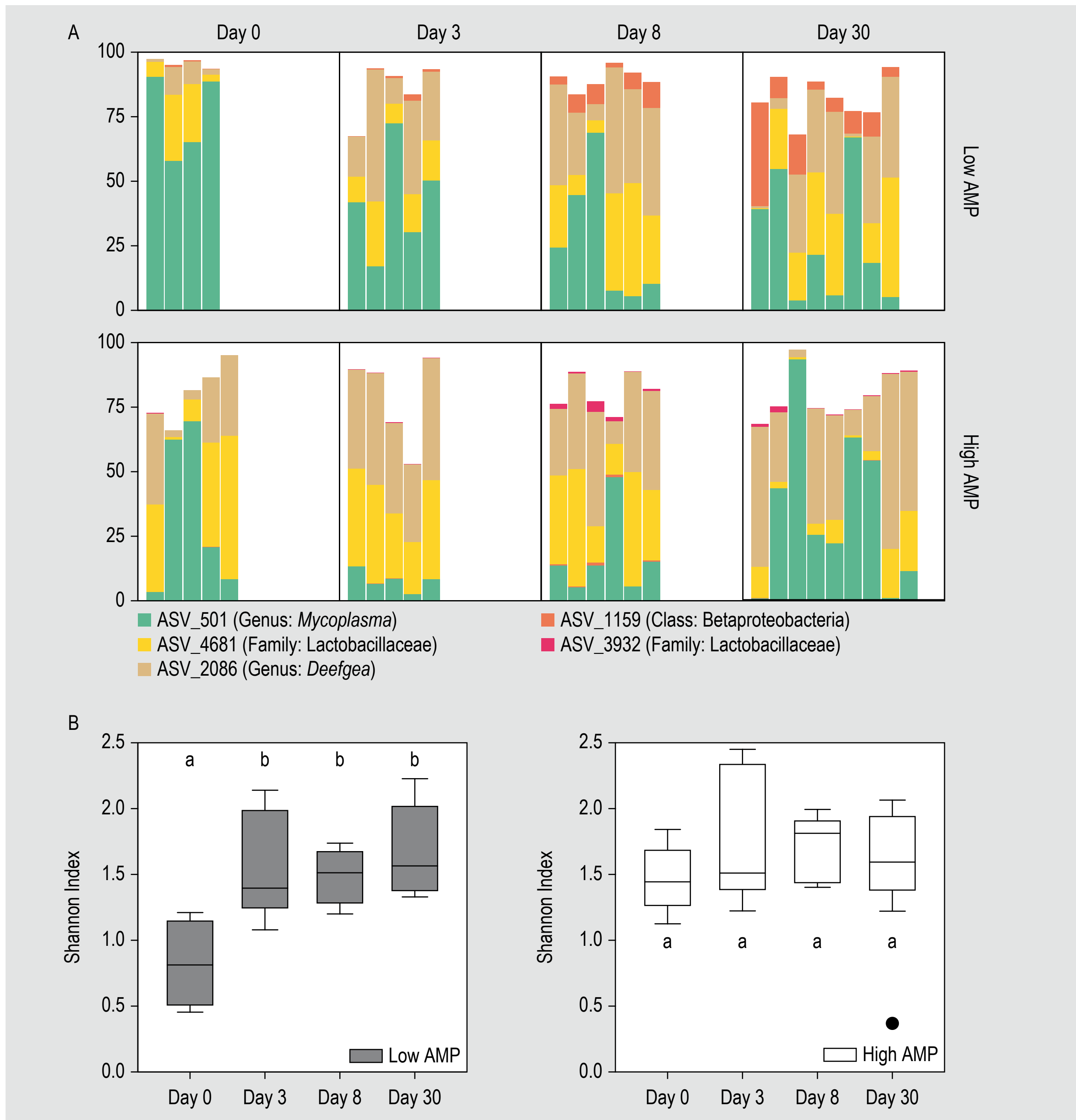

Figure 5. Microbiomes of mock-challenged fish fed either the low antimicrobial peptide (AMP) or high AMP insect diet. (A) Relative abundances of the five most prevalent ASVs in fish intestinal DNA samples over time. The microbiomes of mock-challenged fish were broadly similar between diet groups and over time. Each diet group had a unique member of the Lactobacillaceae family; these strains were likely part of the microbiomes of the flies used to generate the diets. (B) Internal (alpha) diversity of each diet group is shown over time, as measured by Shannon index. Data for each diet group was analysed separately by ANOVA, with post-hoc Tukey test: groups with different letters have statistically significant different mean values. ASV = amplicon sequence variants.

fish. DESeq2 analysis revealed a single ASV significantly enriched (adjusted P-value<0.05) over the 30-day feeding period in each diet group; both of these strains were mapped to the family Lactobacillaceae (Supplementary Figure S3, Supplementary Table S3).

Differential modulation of the intestinal microbiomes of challenged fish fed the low and high AMP diets

Immersion challenge with *Y. ruckeri* resulted in shifts in the microbiome over the course of infection and recovery (Figure 6A). Prior to challenge, microbiomes tended to

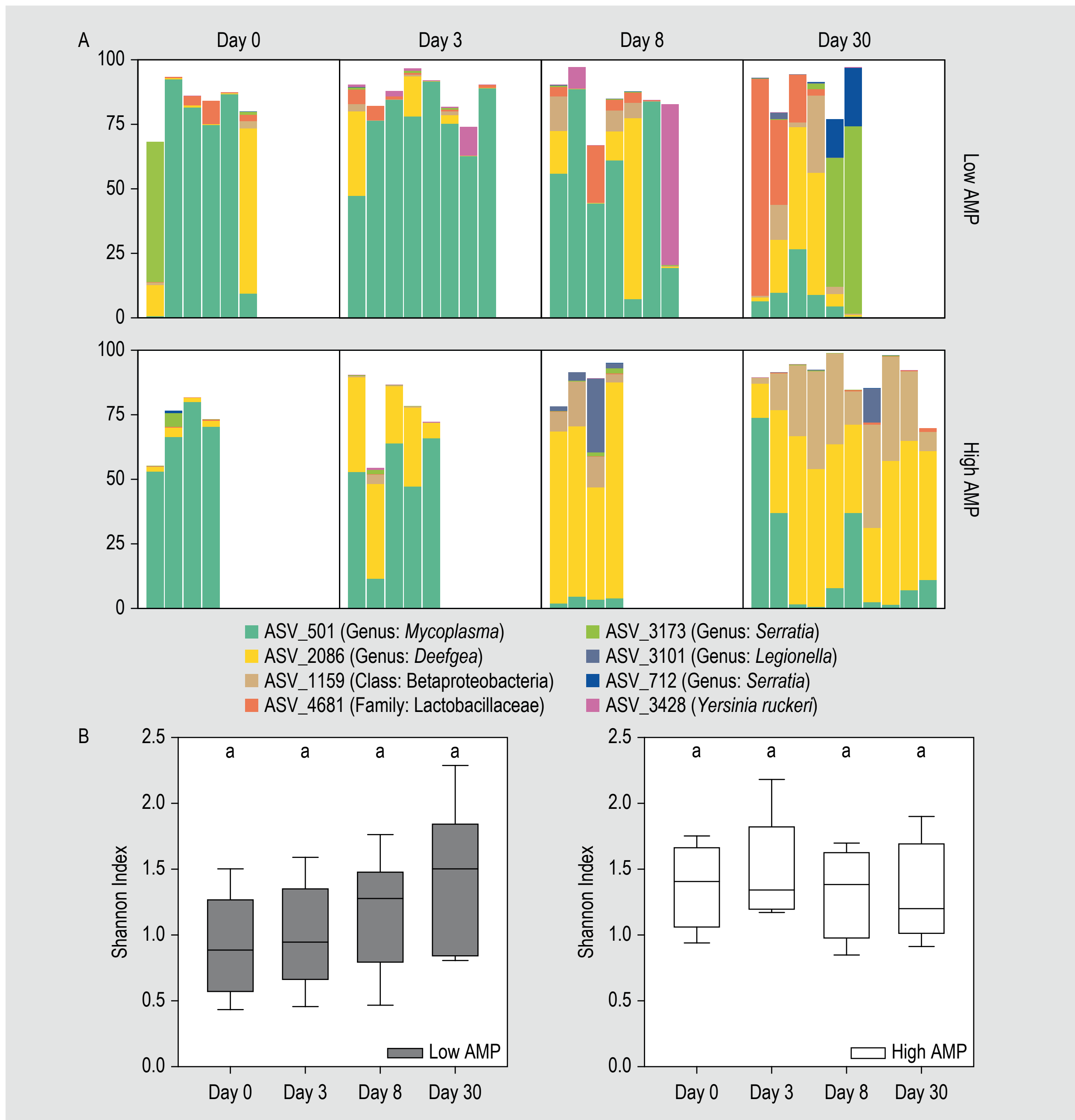

Figure 6. Microbiomes of fish challenged with *Yersinia ruckeri* and fed either the low antimicrobial peptide (AMP) or high AMP insect diet. (A) Relative abundances of the eight most prevalent amplicon sequence variants (ASVs) over time by diet group. The highest relative abundances of *Y. ruckeri* occurred in fish fed the Low AMP diet. Microbiomes shifted over time in both diet groups, with *Mycoplasma* sp. being replaced as the most abundant ASV in most fish by day 30. Microbiomes were significantly different between diet groups at Day 3, Day 8, and Day 30 by PERMANOVA (adjusted *P*<0.05). (B) Internal (alpha) diversity of each diet group is shown over time, as measured by Shannon index. Data for each diet group was analysed separately by ANOVA: no significant differences over time were detected for either diet group.

display high levels of *Mycoplasma* sp. After the challenge, *Mycoplasma* sp. relative abundance dropped in the high AMP diet group and was largely replaced by *Deefgea* sp. and, to a lesser extent, an ASV from the class Betaproteobacteria. In the low AMP diet, *Mycoplasma* sp. levels remained high at day 3, began to decline somewhat by day 8, and were low at day 30. At days 3, 8, and 30, microbiomes of challenged fish were significantly different between the diet groups (PERMANOVA, adjusted-*P*<0.05). The microbiomes of fish fed the low AMP diet were more variable, with higher intragroup beta-diversity at day 8 (mean Bray distance: 0.63) and day 30 (0.75) than fish fed the high AMP diet at the

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corresponding timepoints (mean Bray distances: 0.32 and 0.41, respectively). Consistent with this finding, a greater number of different ASVs had high relative abundances in the microbiomes of fish in the low AMP diet group. These included *Serratia* sp., the Lactobacillaceae sp. associated with this diet group, and *Y. ruckeri*. Despite these shifts in microbial composition, alpha diversity levels were not significantly different between timepoints for either diet group (Figure 6B). In addition to the Lactobacillaceae strains identified in mock-challenged fish, two additional enriched ASVs were identified by DESeq2 – both in the low AMP diet group: *Rhodobacter* sp., and a member of the family Enterobacteriaceae (Supplementary Figure S4, Supplementary Table S4).

4. Discussion and conclusions

This study aimed to evaluate the effects of insect-based diets with different levels of antimicrobial peptides (AMPs) on fish subjected to a bacterial infection challenge. We utilised a genetic model of immune regulation to vary the transcription of AMPs in *Drosophila* and then produced feeds using insect meal made from these flies. In this study, the primary focus was on detection of direct antimicrobial effects of these diets *in vivo*. To that end, the presence and abundance of *Y. ruckeri* in the fish intestine was monitored over time and the microbiomes of fish in both infection challenge and mock-challenge settings were analysed. In mock-challenged fish, no differences in overall microbiome structure between the high and low AMP diet groups were observed. In challenged fish, however, there were multiple trends suggesting that the high AMP diet group may have been beneficial: higher survival, lower median loads of *Y. ruckeri*, and a more stable microbiome composition. It should be noted that all of these observations suffer from a lack of statistical power; our results suggest that elevating AMP levels in insect meals is a promising direction for future research, but no firm conclusions should be drawn from this data.

Many of the limitations of this study stem from technical challenges of producing *Drosophila* in sufficient quantities for production of experimental feed. The first point that should be noted is that the *Drosophila* insect meals were below previously reported protein levels, and the diets were therefore below the published protein requirement of 39.5% for rainbow trout of this size (National Research Council, 2011). This could explain why the survival rates in the ill-fated first challenge trial were lower than expected for both diet groups; fish from the same cohort challenged at the same time and fed a nutritionally complete diet as part of a separate study had higher survival rates (Sibinga *et al.*, 2022). The nutritional profiles of the *Drosophila* meals were similar to one another, however, and we observed no ill-effects of either diet in mock-challenged fish. Therefore, we believe that comparison between the diet groups in the context of this study is feasible, though admittedly not ideal. In the second challenge trial, there was a trend towards higher survival in fish fed the high AMP insect diet, but this was not statistically significant. Similarly, the high AMP diet appeared to decrease the load of *Y. ruckeri* in the intestines of challenged fish, but this again was not significant. These are intriguing results but should be repeated with larger sample sizes of fish and nutritionally complete diets. Use of genetically engineered production insect species (e.g. black soldier flies) in future studies would likely help to resolve many of these issues; thankfully the tools for such an approach seem likely to become more widely available in the coming years (Zhan *et. al*, 2020).

It is likely that the AMP levels present in the high AMP diet in this study were not as high as those of previously reported diets using purified AMPs. We observed that flies from the high AMP (*HS_imd*) *Drosophila* line had approximately 24-fold upregulated expression *cecropin c* compared to flies from the low AMP (*Rel*$^{-}$) *Drosophila* line. Because of the promoter system in this line, heat-shocking the high AMP flies further increased expression to over 100-fold upregulation. However, incubation at 37 °C to induce the heat shock response weakened flies and led many to perish in the softened media; the decision was therefore made not to use heat-shocked flies. There remains potential for higher levels of AMPs than those achieved in this study by using either a different promoter system or an improved methodology for heat shocking large numbers of flies. Furthermore, regulation at the level of the Imd pathway is a relatively blunt instrument; there is an entire suite of AMPs regulated by the Toll pathway that merits investigation. New *Drosophila* models are also capable of manipulating expression levels of individual AMPs (Hanson *et al.*, 2019), which could allow for more precise testing of the effects of insect AMPs on fish intestinal microbiome and resistance to disease.

We have previously demonstrated that the inclusion of a purified insect AMP in the diet can alter the rainbow trout microbiome by reducing alpha diversity and selecting against gram-negative bacteria (Sibinga *et al.*, 2022). Other studies have shown protective effects of dietary insect AMPs against bacterial infection in piglets (Wu *et al.*, 2012), broilers (Zhou *et al.*, 2014), and fish (Dai *et al.*, 2020). Furthermore, recent studies have manipulated AMP levels of insect products by altering the feed substrate (Zhang *et al.*, 2022) or mimicking bacterial infection (Hwang *et al.*, 2022). For the moment, however, it is exceedingly difficult to compare AMP levels across these different kinds of studies. Quantitative measures of AMP levels in processed insect ingredients are lacking; most published comparisons of AMP levels in insects are inferred either from RNA transcription data or from antimicrobial activity of extracts from a relatively small number of individuals insects. This makes it difficult to assess the relevance of the

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wide range of purified AMP doses present in the animal feed literature. One of the few studies to attempt to bridge this gap compared antimicrobial activity levels of immune-stimulated *Drosophila* haemolymph to those of purified solutions of cecropin (Samakovlis *et al.*, 1990). Based on the findings of that study and literature values for size (Church and Robertson, 1966; Katz and Young, 1975) and haemolymph content (MacMillan and Hughson, 2014) of *Drosophila* adults, we estimate the total antimicrobial activity of immune-stimulated *Drosophila* haemolymph to be very approximately equivalent to 120 mg cecropin per kg (dry weight) of flies. At the 20% inclusion level of insect meal present in this study, this would equate to ~24 mg/kg of feed – below the levels reported in experimental diets for fish (75-250 mg), but not hopelessly so. It should be emphasised, however, that this is an incredibly rough estimate based on data cobbled together from several studies and only reflects the contributions of haemolymph AMPs. Furthermore, although in this study flies were flash frozen and lyophilised and diets were similarly produced to try to minimise degradation of AMPs (Izutsu, 2014), there is significant potential for loss of endogenous antimicrobial activity during insect processing. Increased availability and accuracy of proteomic analyses will hopefully provide more accurate information on the total amount of AMPs present in insects, insect meals, and finished animal feeds, though this kind of analysis is not trivial (Dong *et al.*, 2021).

The final key limitation of this study is the presence of confounding variables for analysis of the fish microbiomes. Microbiomes typically shift in response to changes in diet (Ringø *et al.*, 2016), and this effect would potentially be exacerbated by the use of nutritionally deficient diets. These effects, however, should be consistent between the tested diets; of more concern are the confounders of diet-specific effects. We did not use germ-free (axenic) flies and we did not sequence the fly microbiomes. It is probable that viable bacteria remained in the feed and established themselves in the fish gut; since these fly lines came from different source populations and were reared separately, it is also likely that they had different microbiomes. In assessing the changes to fish microbiomes there are therefore two key experimental factors to consider: the microbes introduced directly in the feed from each line of flies and the selection pressure due to AMPs. While better study design would be necessary to conclusively separate these factors, we can make inferences. *Lactobacillus* species tend to be abundant in the microbiomes of *Drosophila* reared on cornmeal media (Douglas, 2018). Since ASVs 4681 and 3932 are *Lactobacillus* spp. that are specific to each diet group, we propose that these were dominant microbial taxa of the low AMP and high AMP *Drosophila* lines, respectively. In the fish, these were the taxa most significantly enriched by each diet, and the only significantly different taxa between diet groups of mock-challenged fish identified by DESeq2. This suggests minimal effects of the AMP levels present in these diets on the fish microbiome in mock-challenged fish. In challenged fish, however, the microbiomes of the two diet groups were more divergent. At the day 3 and day 8 time points, challenged fish in the low AMP diet group had higher mean relative abundance of ASV 501 (*Mycoplasma* sp.), which has been proposed as a biomarker of health (Bozzi *et al.*, 2021); by day 30, however, relative abundance of *Mycoplasma* was similar between the groups. Challenged fish in the low AMP diet group may have been more prone to carry *Y. ruckeri* or ASVs from the genus *Serratia*, members of which have previously been identified as opportunistic pathogens of fish (Baya *et al.*, 1992; McIntosh and Austin, 1990). This is limited evidence, however, and there remains insufficient knowledge of fish microbiomes to confidently differentiate between healthy and unhealthy microbiomes (Legrand *et al.*, 2020). Overall, although the microbiomes of challenged fish were significantly different between diet groups at day 30, the effect of dietary AMP levels on the fish microbiomes in this study is difficult to characterise.

The size and collective expertise of the *Drosophila* research community is a tremendous resource for the study of farmed insect species. The current study hopefully sheds light on some of the experimental approaches unlocked by these tools and perspectives, while also highlighting the challenges of translating science in *Drosophila* to farmed insects; the small size of *Drosophila* makes them an especially challenging species for production of experimental feeds. Fortunately, annotated genomes for mealworms (Eriksson *et al.*, 2020) and black soldier flies (Generalovic *et al.*, 2021) and new CRISPR/Cas9 tools for gene editing in these species (Zhan *et al.*, 2020) mean that many of the tools developed in *Drosophila* labs should hopefully be adaptable to production insect species in the coming decade. There is ample opportunity for knowledge transfer and collaboration between these fields.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2022.0132.

Figure S1. Expression levels of the AMPs *cecropin c* and *drosomycin* by *Drosophila* line and heat-shock treatment.

Figure S2. Expression levels of *cecropin c* over the collection period.

Figure S3. Volcano plot showing taxa enriched in microbiomes of fish fed diets produced from either high- or low-AMP insect meals 30 days after mock-challenge.

Figure S4. Volcano plot showing taxa enriched in microbiomes of fish fed diets produced from either high-

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or low-AMP insect meals 30 days after challenge with *Yersinia ruckeri*.

Table S1. Primer sequences.

Table S2. Samples sequenced and retained after quality control.

Table S3. DESeq2 analysis results for mock-challenged fish, day 30 timepoint.

Table S4. DESeq2 analysis results for *Yersinia ruckeri* challenged fish, day 30 timepoint.

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Disclaimer

Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

Conflict of interest

The authors declare no conflict of interest.

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