Energetic costs of mounting an immune response in a coral reef damselfish (*Pomacentrus amboinensis*)

Marie Levet[1], Dominique G. Roche[2, 5], Shaun S. Killen [1,3], Simona Colosio[2], Redouan Bshary[2], Joanna J. Miest[4, 6], Sandra A. Binning[1,2]

[1] Groupe de recherche interuniversitaire en limnologie (GRIL), Département de sciences biologiques, Université de Montréal,1375 Av ; Thérèse-Lavoie-Roux, Montréal, QC, Canada, H2V 0B3

[2] Institut de Biologie, Éco-éthologie, Université de Neuchâtel, Neuchâtel 2000, Switzerland

[3] School of Biodiversity, One Health and Veterinary Medicine,Graham Kerr Building, University of Glasgow, Glasgow, Scotland, United Kingdom, G12 8QQ

[4] School of Science, University of Greenwich, Chatham Maritime, Kent, United Kingdom ME4 4TB

[5] Department of Biology and Institute of Environmental and Interdisciplinary Sciences, Carleton University, Ottawa, ON, Canada, K1S 5B6

[6] Natural and Applied Sciences, Canterbury Christ Church University, Canterbury, Kent, United Kingdom, CT1 1QU

* corresponding author: marie levet; marie.levet@umontreal.ca

Abstract

While immune responses can be energetically costly, quantifying these costs is challenging. We tested the metabolic costs of immune activation in damselfish (*Pomacentrus amboinensis* Bleeker, 1868) following a mass-adjusted injection of lipopolysaccharide (LPS) endotoxin. Fish were divided into eight treatments: two controls (handling and saline injection) and six LPS groups with concentrations ranging from 3 to 100mg kg^{-1}. We used intermittent flow respirometry to measure differences in oxygen uptake (delta$\dot{M}O_2$) 20h before versus 20h after LPS injection and changes in metabolic traits (lowest, routine and peak metabolic rates) as proxies of the aerobic costs of metabolism. Spleen somatic index (SSI) and gene expression in spleens were measured to assess immune activation. We found no difference in metabolic traits or SSI but observed different non-linear patterns of delta$\dot{M}O_2$ in fish exposed to 50 and 100mg kg^{-1} LPS compared to lower doses and controls. Fish exposed to high doses of LPS also had lower residual aerobic scope compared to controls and lower LPS doses. Fish exposed to doses of 3, 50, and 100mg kg^{-1} showed altered gene expression compared to the handling control. Overall, our results suggest that immune activation has measurable effects on metabolic traits that are both dose and time-dependent.

Keywords

Pomacentrus amboinensis, metabolism, gene expression, spleen somatic index, generalized additive mixed model, damselfish

Introduction

Infection with macroparasites such as helminths and arthropods and/or microparasites such as bacteria, protozoa, fungi or viruses can be costly for hosts and decreases host fitness (Poulin and Morand, 2000; Schimdt-Hempel 2003; Goater et al. 2014). In animals, infection is often

linked to the activation of the host's immune system. Upon recognition of a disease-causing agent, the host's innate immune system, which is responsible for non-specific immune responses, activated along with a systemic inflammatory response and activation of the hypothalamic-pituitary-adrenal axis; together, these form part of the acute phase response (APR) to infection (Magnadottír 2010). The APR triggers a series of cellular reactions including hormone and cytokine secretion; activation of lymphocyte differentiation (B-cells) and proliferation (B cells and T-cells) as well as secretion of plasma protein in the liver (Møller and Erritzoe 2002; Reyes-Cerpa et al. 2012) resulting in an increase in metabolic demands. For example, mosquitofish (*Gambusio holbrooki* Girard, 1959) can experience a significant reduction in body mass while expressing an immune response, suggesting the use of energy reserves to sustain an increase in metabolic demands due to immune activation (Bonneaud et al. 2016). The metabolic costs of immune activation can also be quantified by estimating changes in aerobic metabolism (Powell et al. 2005; Methling et al. 2019). For instance, immune-challenged zebra finches (*Taeniopygia guttata* Vieillot, 1817) increase their resting metabolic rate in response to lipopolysaccharide (LPS) injection (Burness et al. 2010). These results suggest a trade-off between energy allocation to immune function and other fitness-enhancing activities (Sheldon and Verhulst 1996; Mills et al. 2010).

Despite some evidence for this trade-off, several studies fail to demonstrate an energetic cost associated with host immune activation. For example, side-blotched lizard (*Uta stransburiana* Baird and Girard, 1852), and wild white-footed mice (*Peromyscus leucopus* (Rafinesque, 1818) showed no changes in their metabolic rates in the days, after being exposed to an immune stimulant (Derting and Compton 2003; Smith et al.2017).

Several non-mutually exclusive factors could explain the inconsistencies in the energetic costs of mounting an immune response observed among studies and species. For example the

methods used to experimentally induce non-pathogenic immune reactions in hosts vary among studies. Studies commonly use phytohaemagglutinin (PHA) or LPS to trigger a host immune response. Yet, each method yields differences in terms of the immune reaction it triggers. Injection of PHA – a non-pathogenic antigen from a plant lectin – induces swelling (Martin et al. 2006). Alternatively, LPS – an endotoxin present in gram-negative bacteria – provokes an inflammatory response characterized by "sickness behaviour", such as fever, lethargy, and reduced physiological function, often leading to a decrease in food intake and/or reproductive success (Bonneaud et al. 2003; Melhado et al. 2020; Lopes et al. 2021). As such, the energetic costs of different forms of immune stimulation may differ. For example, fish-eating myotis bats (*Myotis vivesi* Ménégaux, 1901), injected with 50 µL of PHA show no change in body mass or routine metabolic rate in the 54 hours post-injection (Otàlora-Ardila et al. 2016). In contrast, individuals injected with 50 µL of LPS experience a decrease in body mass and a drastic increase in their routine metabolic rate (up by 140-185%) within 11 hours post-injection (Otàlora-Ardila et al. 2016). This discrepancy suggests a higher energetic cost associated with the physiological reactions induced by LPS compared with PHA.

Another factor that may contribute to discrepencies among studies examining the energetic costs of the immune response is that immune responses are species-specific, regardless of the immune stimulant used to induce immune activation (Berczi et al. 1966; Martin, Weil, and Nelson 2007). For instance, mammals and birds are extremely sensitive to endotoxins and react strongly to LPS injection (Berczì et al. 1966; Hasselquist and Nilsson 2012). For guinea pigs, (*Cavia porcellus* (Linnaeus,1758)), an injection of 10mg kg^{-1} of LPS can lead to lethal endotoxin shock. In contrast, some teleost fishes are known to be remarkably tolerant to LPS, with doses of 50mg kg^{-1} of LPS failing to induce sickness symptoms in coho salmon (*Oncorhynhus kisutch* (Walbaum,1792)) or rainbow trout (*Salmo gaidneri* (Walbaum,1792)

(Berczì et al. 1966). It is noteworthy that most studies exploring the physiological costs of immune activation focus on birds and mammals (Hasselquist and Nelson 2012). Similar studies in reptiles, amphibians or fishes remain scarce despite known differences among taxa and species (Ackerman et al. 2000; Mackenzie et al. 2006; Zanuzzo et al. 2015; Bonneaud et al. 2016; Bennoit and Craig 2020). Finally, experiments often study the effects of a single dose, or limited doses, of a given immune stimulant without the necessary controls (i.e., a handling control and a saline injection control), making it difficult to understand the dose-response of hosts to endotoxin administration. Individual within species variation in metabolic rates are also not typically considered in study designs despite minimum metabolic rates sometimes varying greatly (2-3-fold) among individuals within the same population (Burton et al. 2011). This large among-individual variation in metabolic rates makes it more difficult to detect differences among treatments.

Here, we examine the metabolic costs of mounting an immune response in the coral reef damselfish, (*Pomacentrus amboinensis* Bleeker, 1868), by measuring oxygen uptake in individuals pre- and post-immune stimulation, across a range of LPS doses. We also assess differences in spleen mass and gene expression across treatments to assess the effectiveness of an endotoxin injection in stimulating an immune response. The spleen is a major lymphoid organ in fish and plays a critical role in clearing infection with spleen size often used as an indicator of an immune response in fish (Lefebvre et al. 2004; Rauta et al. 2012). *Pomacentrus amboinensis* are well-studied in terms of their physiology and behaviour. This species was previously found to mount an immune response following injection of 50mg kg^{-1} of LPS as demonstrated by an alteration in gene expression associated with immune activation (Binning et al. 2018). Thus, our objectives were to (1) assess whether individuals respond physiologically to an immune challenge with LPS through changes in oxygen uptake, spleen size and alteration

126 in immune gene expression; and if so, (2) determine whether the host's immune response varies
127 across LPS doses. We hypothesized that higher doses of LPS would cause the most significant
128 difference in oxygen uptake pre- and post-injection. Additionally, we hypothesized that fish
129 exposed to LPS would have a higher spleen somatic index (SSI) and an altered immune gene
130 expression compared to both control groups (saline injection and handling control) if the
131 injection triggers an immune response.
132
133 **Methods**
134 **Fish collection and husbandry**
135 This study was conducted at the Lizard Island Research Station (LIRS), on the northern Great
136 Barrier Reef, Australia (14° 40' S;145° 28' E) within a two month period between July and
137 September 2015. Field collections and experiments were conducted under permits from the
138 Great Barrier Reef Marine Park Authority (G14/37048.1) with approval from the Queensland
139 Government (DAFF) Animal Ethics Committee (CA 2015/07/878). A total of forty-eight
140 females *P. amboinensis* were collected on July 25th and August 13th (non reproductive period)
141 using hand nets and monofilament barrier nets (10 mm stretch mesh) from continuous reef sites
142 in the Lizard Island lagoon (3-5m depth) by two divers on SCUBA.
143 Fish were transported in individual water-filled plastic Ziploc bags to the aquarium facilities at
144 LIRS within 90 minutes of capture. To remove unencysted ecto- and endoparasites, fish were
145 treated with a Praziquantel solution (1 tablet dissolved in 3ml ethanol, mixed in 20L water) for
146 90 minutes, followed by 60 seconds in a freshwater bath. Each individual was then measured
147 (mass (M), , standard length (SL)) and transferred to an individual white holding aquarium
148 (28.0 W X 38.0 L X 18.0 H) with an opaque PVC tube (6.0 cm L X 5.5 cm diameter) for shelter.
149 Fish were fed Nutrafin Tropical fish flakes twice a day to satiation.
150

Respirometry measurements – estimate of metabolic traits

Metabolic rates for all forty-eight individuals (SL: 52.2 ± 5.7 mm; M: 5.9 ± 2.1g; mean ± S.D.) were estimated using automated intermittent-flow respirometry in resting chambers (AquaResp, www.aquaresp.com) fitted with a recirculation loop to allow proper mixing (Roche et al. 2013; Clark et al. 2013; Rosewarne et al. 2016; Killen et al. 2021). We measured an individual's rate of oxygen uptake ($\dot{M}O_2$; mg O_2 h^{-1}) as a proxy for aerobic metabolic rate. Full details of the respirometry setup are given in supplemental material (Table S1), following the reporting guidelines described in Killen et al. (2021). Respirometry trials were carried out in custom made cylindrical 0.5 L resting chambers (volume includes recirculation tubes) submerged in U.V. sterilized, temperature-controlled and aerated water baths with water temperature maintained at 24 ± 0.3°C (actual variation) using a TECO TC 20 water chiller / heater system. Chambers were opaque with only a small viewing window located on the top to minimize visual disturbance to the fish. Dissolved oxygen concentration was recorded with a four channel FireSting O_2 Optical Oxygen Meter (Pyroscience, Aachen, Germany). Every six days, the respirometry chambers were disassembled, rinsed in freshwater and left to dry for 8h to reduce background oxygen consumption due to bacteria. Changes in water oxygen tension (PwO_2) due to fish respiration were continuously monitored at 1 Hz with the software AquaResp.

All respirometry trials began between 17.00 and 19.00 with an exhaustive chase protocol followed by air exposure to estimate the maximum metabolic rate (hereafter: MMR), the upper limit of a fish capacity to aerobically metabolize oxygen under a given environmental condition (Norin and Clark 2016; Killen et al. 2017). To estimate MMR, fish were individually placed in a circular bucket (30 cm diameter) and chased continuously by hand for three minutes, held out of the water for one minute and after that, then immediately placed in the respirometry chambers (Roche et al. 2013). The first measurement period commenced when the respirometer was

sealed (maximum 15 seconds). For this first measurement, the chamber remained closed for 15 minutes to have continuous measurements to calculate $\dot{M}O_{2max}$. Afterwards, ten minutes loops with a 240s flush, 60s wait and 300s measure cycle were taken to measure $\dot{M}O_2$. This continuous periodic monitoring of $\dot{M}O_2$ in the respirometer allowed estimation of individual standard metabolic rate, the minimum energy required in a post-absorptive state to maintain homeostasis, overnight (hereafter: SMR) (Chabot et al. 2016). We calculated SMR as the 20^{th} percentile of measures taken overnight, excluding the first five hours after transfer to the respirometer to minimise the effects of stress on estimates of SMR (Chabot et al. 2016). From these measures, we were able to determine individual's aerobic scope (hereafter: AS), the total amount of energy available to allocate to physiological systems including the immune system, calculated as MMR-SMR (Derting and Compton 2003; Claireaux and Lefrançois 2007; Chabot et al. 2016*b*). We fasted the fish for for 24h prior to the respirometry trials to avoid increases in $\dot{M}O_2$ caused by a digestive state (Jobling 1981, Chabot et al. 2016*b*). The body mass of each fish was determined to the nearest 0.1g before the start of each trial. Two loops were run without a fish in the respirometer to measure initial and final background rates of respiration. Background respiration was accounted for by subtracting oxygen consumption in an empty chamber from concurrent $\dot{M}O_2$ values taken in chambers containing fish (Rodgers et al. 2016). Artificial lighting was turned off, and only natural lighting was available during the rest of the trial.

Respirometry - Immune challenge

After approximately 20h in the respirometer, fish were given an immune challenge to estimate the metabolic costs of mounting an innate immune response. We used lipopolysaccharide (LPS; Sigma-Aldrich L2880, serotype 055:B5), a component of the *E*-coli cell wall, to elicit an immune response in fish. Fish were haphazardly assigned to one of eight treatments (6 fish per treatment) such that the mean fish mass was similar among treatment groups: 1) handling

control, 2) saline injection (0.9 % saline control), 3) 3mg kg^{-1} LPS, 4) 5mg kg^{-1} LPS, 5) 10mg kg^{-1} LPS, 6) 30mg kg^{-1} LPS, 7) 50mg kg^{-1} LPS, 8) 100mg kg^{-1} LPS. Fish were removed from the respirometers and injected intraperitoneally (i.e. abdominal cavity) using heparinized 29 gauge insulin syringes (heparin concentration: 28 mg/ml^{-1}) through a plastic bag partially filled with seawater to avoid stress caused by air exposure and to protect the fish from surface abrasions during handling. The volume of the injection was mass-adjusted for each fish resulting in a injected volume standardized across concentrations and fish. Following injection, the test fish was immediately placed back in the respirometry chamber and left for a further 20h to measure $\dot{M}O_2$ post-injection. These 20h of measurements post-injection were taken over the same time period as the period of measurements pre-injection. With post-injection $\dot{M}O_2$ values, we measured the peak, lowest, and routine metabolic rate to compare difference in metabolic traits among groups. We defined the lowest metabolic rate as the 20^{th} percentile of $\dot{M}O_2$ values post-injection and calculated the routine metabolic rate as the average $\dot{M}O_2$ values post-injection. We selected the peak value of $\dot{M}O_2$ as a proxy for the peak metabolic rate post-injection. Furthermore, we calculated the percentage of aerobic scope available for the fish across time within each treatment by subtracting $\dot{M}O_2$ values post-injection from the aerobic scope calculated pre-injection. We also estimated the total integrated post-injection oxygen uptake for each individual by calculating the area under the curve (AUC) of $\dot{M}O_2$ versus time post-injection. AUC was calculated as integral of $\dot{M}O_2$ post-injection using the trapezoid rule (Chabot et al. 2016*b*). A positive AUC suggests an overall increase in energy demands following injection, whereas a negative AUC suggests a decrease in energy demands following injection. Post-respirometry experiments, the fish were returned to their holding tanks for a week and fed twice per day.

Tissue collection

To ensure sufficient time for the systemic and the adaptive immune response to establish a seven day period was observed between the LPS injection and the tissue collection. This time period is sufficient for changes in gene expression to be detected in this species (Binning et al. 2018). Fish were euthanized in an overdose of AQUI-S® solution (AQUI-S New-Zeland Ltd). Fish weight and length were measured again. The spleen was then dissected out and weighed. Spleen tissue was preserved in RNAlater® (R0901 Sigma-Aldrich), frozen, and stored at -20°C until they were shipped to the University of Greenwich for PCR analysis.

Spleen tissue and gene expression analysis

The spleen somatic index (SSI) was calculated as the proportion of the spleen in relation to the total fish wet mass. The protocol used for immune gene analysis is described in Binning et al. (2018). Briefly, RNA was extracted from the spleen with ReliaPrep™ RNA Cell Miniprep System (Promega). RNA concentration was determined using a nanodrop 2000 (Thermo Scientific). The samples were then normalized to a common concentration of 50ng μl^{-1} using nuclease-free water (Promega). 425 ng RNA were treated with DNAse (Quanta PerfeCta DNase I Kit (QuantaBio)) and 212 ng of the treated RNA were transcribed using Quanta qScript cDNA Synthesis Kit (QuantaBio) with a total volume of 10 µl following manufacturer instructions. A 1:10 dilution with Nuclease-free water (Promega) was performed and samples were stored at 4°C overnight. Gene expression analysis was performed the next day. Immunological response to LPS challenge was quantified using gene polydom/svep1 (Binning et al. 2018). To ensure specificity melting curves of the qPCR products were checked between 60 and 90°C with 1°C intervals. Gene expression data (ct-values) were normalised against the house keeping gene 12s, to give dct. One fish in the LPS30 was not included in the SSI and gene expression analysis due to an inability to retrieve the spleen. Two fish in the handling control, one fish in the saline group, one fish exposed to 3mg $kg^{-1}$of LPS, one fish exposed to 5mg kg^{-1} of LPS, and one fish

exposed to 100mg kg^{-1} of LPS had a spleen too small to perform RNA extraction preventing the analysis of immune gene expression.

Statistical analysis

All statistical analyses and data visualization were performed using R v.4.0.3 (R Core Team, 2016). We examined the relationship between metabolic traits (lowest, routine, and peak metabolic rates) and treatment with a linear model (LM). The lowest, routine, and peak metabolic rates were used as response variables, with treatment, fish mass and their interaction as predictors. This allowed us to assess group differences in metabolic traits while accounting for mass as a covariate. Body mass and metabolic rates were both $\log_{10}$ -transformed to normalize the data. Percentage of residual aerobic scope available at each time step was calculated for each fish by estimating individual AS pre-injection, from which we subtracted the AS estimated post-injection. Then, we compared the average available aerobic scope among treatments. Values above 100% indicate that oxygen uptake post-injection was lower than pre-injection whilst values below 100% indicate an increase in oxygen uptake post-injection. To observe changes in the $\dot{M}O_2$ patterns among treatments through time, we measured the difference in $\dot{M}O_2$ (delta$\dot{M}O_2$). We calculated delta$\dot{M}O_2$ by subtracting the $\dot{M}O_2$ pre-injection from the $\dot{M}O_2$ post-injection taken at the exact same time. For example, fish $\dot{M}O_2$ value taken pre-injection at 7:00:00 pm were subtracted to its $\dot{M}O_2$ value taken post-injection at 7:00:00 pm the following day. By doing so, we aimed to determine the change in an individual's oxygen uptake relative to its own baseline over time. The first hour of $\dot{M}O_2$ measurement pre- and post-injection were excluded from $\dot{M}O_2$ measurement to exclude any change in oxygen uptake linked to either, the MMR measurement pre-injection or the handling/injection post-injection (Fig.S1). To capture the nonlinear pattern observed for changes in $\dot{M}O_2$ over time among treatments, we used a generalized additive mixed model (GAMM; function "gam" in R package "mgcv", Wood

and Wood 2015). GAMMs are an extension of generalized linear mixed models using smooth terms alongside parametric terms. Smooth terms are a set of non-linear functions (i.e., basis functions), which are multiplied each by a coefficient estimated from the data and summed. Using the gam.check () function, we determined the number of basis functions needed for the smooth term "time" in our model. The k parameter (i.e., number of basis functions) was set to 30 basis functions for the smooth term "time". Our model contained the parametric terms "delta$\dot{M}O_2$" (response variable), "treatment" (explanatory variable), "mass" (explanatory variable), and the smooth term "time" (explanatory variable) representing patterns related to the non-linear change in delta$\dot{M}O_2$ over time (in minutes). We included the random effect 'fish ID' in the model to allow smoothers to vary in intercept and slope. We used restricted maximum likelihood (REML) for parameter estimation. Following diagnostic plots assessment, we used the scaled *t* family in place of Gaussian as our data appeared heavy tailed (Wood 2017).

We used estimation statistics to determine differences in the spleen somatic index (SSI), immune gene expression, group mean, standard deviations and treatment effect sizes with 95% confidence intervals are presented using Gardner-Altman estimation plots (*R package* " dabestr", Ho et al. 2019).

We used a Bayesian multivariate model (*R package MCMCglmm*; Hadfield et al. 2010) with a Gaussian error distribution to examine covariation among three immune activation parameters: SSI, immune gene expression, and the AUC. Treatment and fish mass were included in the model as fixed effects. Predictor variables were mean-centered (mean = 0) and standardised to one standard deviation. Visual inspection of diagnostic plots (parameters traces) indicated chain convergence. All statistical analyses and data visualization were performed using R v. 4.0.3 (R Core Team, 2016).

Results

Change in metabolic traits post-injection

We found no significant difference in the peak metabolic rate attained by fish across groups post-injection (LM, $F_{(7,32)} = 0.93$, $p = 0.491$) (Fig.1 (a)). There were also no differences in the lowest metabolic rate (LM, $F_{(7,32)} = 1.130$, $p = 0.369$) and routine metabolic rate (LM, $F_{(7,32)} = 0.76$, $p = 0.62$) (Fig.1 (b-c)). The treatment-mass interaction was not significant for any of the metabolic traits (peak metabolic rate: $F_{(7,32)} = 0.802$, $p = 0.591$; lowest metabolic rate: $F_{(7,32)} = 1.120$, $p = 0.374$; routine metabolic rate: $F_{(7,32)} = 0.605$, $p = 0.746$). Fish mass had a significant positive effect on fish routine metabolic rate ($F_{(7,32)} = 7.150$, $p = 0.011$) and was marginally non-significant for both the peak and lowest fish metabolic rate (repectively: $F_{(7,32)} = 4.053$, $p = 0.052$; $F_{(7,32)} = 4.088$, $p = 0.051$). Fish exposed to 50mg kg^{-1} and 100mg kg^{-1} had, on average, the lowest aerobic scope post-injection with, respectively, 92.57 % and 95.96% of their aerobic scope available (Fig.2 (g-h). In comparison, both control groups approached nearly 100% of their aerobic scope (99,30% for the handling control and 100.26% for the saline) post-injection (Fig.2 (a-b)).Fish injected with doses of 30mg kg^{-1} and lower, all had greater than 95% of their aerobic scope available post-injection (dose of 3mg kg^{-1} = 96.35%; dose of 5mg kg^{-1} = 96,84%; dose of 10mg kg^{-1} = 98.09%; dose of 30mg kg^{-1} = 102.02%) (Fig.2 (c-f)). The peak level of $\dot{M}O_2$ post injection occurred between 11 and 12h for the control groups and the LPS doses ranging from 3mg kg^{-1} to 30mg kg^{-1} (Fig.2 (a-f)). For fish exposed to 50mg kg^{-1} and 100mg kg^{-1}, the peak $\dot{M}O_2$ occurred between 8 and 9 h post injection (Fig.2 (g-h)). Finally, control groups and LPS injected fish showed no difference in AUC (Table 2, Fig.3).

Changes in oxygen uptake overtime

In our GAMM model, the estimated degrees of freedom ranged from 12.30 to 23.22 suggesting a highly nonlinear relationship between the oxygen uptake through time and LPS doses (Table 1). Delta$\dot{M}O_2$ tended to vary through time in fish in both control groups, and in the LPS-

injection treatment groups with dose of 3, 5, 10, and 30mg kg^{-1} (all $p < 0.05$; Table 1, Fig.4 (a-f)) but not in LPS treatment groups with doses of 50, and 100mg kg^{-1} (LPS50: $p = 0.53$ and LPS100: $p = 0.65$; Table 1, Fig.4 (g-h)). In other words, there were changes in $\dot{M}O_2$ uptake in fish exposed to 3, 5, and 10mg kg^{-1} LPS: fish in these groups initally had lower $\dot{M}O_2$ post-injection compared to pre-injection, followed by oxygen uptake above pre-injection values 10 hours post-injection, which occurred around sunrise (time, Fig.4 ((c, e)). This pattern was also observed in fish exposed to a saline injection (Fig.4 (b)). The handling control group and fish injected with an LPS dose of 30mg kg^{-1} showed a similar pattern, exhibiting a steady increase in $\dot{M}O_2$ over time (Fig.4 (a, f)). Interestingly, fish exposed to the two highest LPS doses (50 and 100mg kg^{-1}) had patterns of delta$\dot{M}O_2$ through time that did not differ from 0, suggesting a relatively constant oxygen uptake both pre and post-injection.

Changes in SSI and gene expression

We found no difference in SSI between the two control groups (handling and saline injection) and between the handling control (the contrast group) and the different LPS doses (Table 2, Fig.5). There was no difference in immune gene expression between the handling and saline control groups (Table 2, Fig.6). However, we observed a dose-dependent effect of LPS injection on immune gene expression, with gene expression being altered in fish injected with the lowest dose of LPS (3mg kg^{-1}) and the two highest LPS doses (50mg kg^{-1} and 100mg kg^{-1}) compared to the handling control group (Table 2, Fig.6). There was also an indication of a similar pattern for fish injected with 30mg kg^{-1} of LPS (mean difference in gene expression compared to the handling control: 1.18 dct, 95% *CI*: -0.0031– 3.04).

Covariation between SSI, gene expression, and metabolism

We found no evidence of a strong association between SSI, gene expression, and metabolism following immune stimulation (AUC–SSI r = -0.22; AUC–gene expression r = -0.26; SSI–gene expression r = 0.28; Fig.S2). A more detailed examination of correlations within individual treatment groups supports this finding (Fig.S3 and Fig.S4).

Discussion

Our objectives were to determine if immune stimulated coral reef fish showed difference in metabolic traits and oxygen uptake over time following immune activation and if these differences are dose dependent. We observed no significant difference in metabolic traits post-injection across treatments. However, we found that fish exposed to LPS doses of 3mg kg^{-1}, 5mg kg^{-1}, 10mg kg^{-1} and 30mg kg^{-1} as well as fish in both control treatments had a delta$\dot{M}O_2$ through time relationships that differed significantly from zero. In most cases, delta$\dot{M}O_2$ was lower in the first 10 hours post-injection (night time) followed by a sharp increase in delta$\dot{M}O_2$ around dawn (day time), which remained elevated for the rest of the day. Fish exposed to 50mg kg^{-1} and 100mg kg^{-1} LPS did not follow this trend, and instead tended to have similar delta$\dot{M}O_2$ values both overnight and in the daytime. Fish exposed to these doses of LPS also showed a lower residual aerobic scope on average compared to both controls and lower doses of LPS. We also observed altered immune gene expression in fish injected with the lowest dose (3mg kg^{-1}) as well as the two highest doses (50mg kg^{-1} and 100mg kg^{-1}) compared with fish in the handling control. Fish SSI did not differ across treatments. Overall, these results highlight complex responses to immune system activation that depend on the dose of LPS administered.

Immune stimulation and metabolic traits

Our results are consistent with previous findings observed in ectotherms that showed no difference in metabolic traits following exposure to LPS (Smith et al. 2017; Stahlschmidt and

374 Glass 2020). We hypothesized that triggering an immune response would result in increased
375 oxygen consumption as a result of upregulation in genes associated with immune defense.
376 However, it is possible that an individual may maintain similar metabolic demands and instead
377 reallocate energy away from other activities such as growth and reproduction in order to fuel
378 an immune response (Uller et al. 2006; Lind et al. 2020). Interestingly, across all treatments the
379 remaining aerobic scope was above 90% of that measured pre-injection (Fig.2), suggesting that
380 mounting a mild immune response does not impose a large constraint on the capacity of an
381 individual to perform other aerobic activities simultaneously. Indeed, the overall integrated
382 energy use as measured by the AUC showed no difference among treatments (Table 2; Fig.3).
383 Nonetheless, residual aerobic scope was lowest for the two highest doses of LPS. Indeed,
384 Binning et al. (2018) found that 50mg kg^{-1} of LPS induced an immune response in Ambon
385 damselfish. Whether a 5% or 7% reduction in AS represents a significant cost to fish in natural
386 settings, especially when combined with other biotic or abiotic stressors, remains to be
387 explored.
388
389 An acute inflammatory response from LPS injection can affect host metabolic traits in the
390 minutes to hours post-injection (Novoa et al. 2009; Bonneaud et al. 2016; Otálora-Ardila et al.
391 2016). In the present study doses of 3mg kg^{-1}, 5mg kg^{-1}, and 10mg kg^{-1} elicited $\dot{M}O_2$ responses
392 similar to control treatments. Indeed, oxygen uptake was lower overnight in fish from these
393 groups during the second day in the respirometers, which may be indicative of habituation to
394 the experimental set-up (Fig.4 (a-f)). Delta$\dot{M}O_2$ values in these treatments also trended above 0
395 around 10 hours post-injection, at the onset of dawn, suggesting increased diurnal activity in
396 fish on the second day of experiments. Conversely, fish from the highest-dose treatments (50mg
397 kg^{-1} and 100mg kg^{-1}) tend to have opposite delta$\dot{M}O_2$ trends. Delta$\dot{M}O_2$ values were constantly
398 above 0 within the first 10 hours of the trial before decreasing below or near 0 after 10 hours

399 post-injection. This suggests that these individuals do not show decreased $\dot{M}O_2$ overnight nor
400 increased diurnal activity during the daytime which may be linked to an immune activation
401 beginning within the first 2-4 hours post injection. Otálora-Ardila et al. (2016) observed that
402 time was a key component to consider due to it interacting effect with LPS treatment on the
403 routine metabolic rate of fish-eating bat. Indeed, our results demonstrate that temporal trends
404 in oxygen uptake associated with the magnitude of the immune response can be present despite
405 no significant mean differences in metabolic traits averaged across time. Thus, we believe that
406 constant measures of oxygen uptake over time post immune stimulation is necessary to capture
407 these complex non-linear dynamics.
408
409 The influence of circadian rhythms on fish metabolism is well-documented, and several studies
410 show a tight relationship between variation in oxygen uptake and the light-dark cycle (Svendsen
411 et al. 2014; McKenzie et al. 2015). For instance, lake sturgeon, (*Acipenser fulvescens*
412 Rafinesque, 1817), exhibit variation in $\dot{M}O_2$ over 24 hours, with a peak at dusk, and at dawn,
413 depending on the light cycle the fish are exposed to (Svendsen et al. 2014). The interactions
414 between the circadian cycle and the immune system can lead to increased immune functions
415 during the resting phase (Halberg et al. 1960; Scheiermann et al. 2013). In our experimental
416 timeline, the resting phase occurred directly after the LPS injection (between 0 and 10 hours;
417 Fig.4). We suggest that the increase in oxygen demands above 0 for 50mg kg^{-1} and 100mg kg^{-1}
418 during the fish resting phase is a sign of immune activation. The opposite pattern for delta$\dot{M}O_2$
419 was observed for control groups and doses ranging from 3mg kg^{-1} to 30mg kg^{-1} of LPS with
420 fluctuations in delta$\dot{M}O_2$ below 0 values during the night time and consitently above it after
421 sunrise likely linked to natural biological rhythms of the fish rather than reflecting an immune
422 activation.
423

Immune stimulation effect on the spleen somatic index and gene expression

In fish, the spleen is a crucial secondary lymphoid organ with a central role in systemic immunity due to its functions in coordinating innate and adaptative immune responses (Mebius and Kraal 2005; Uribe et al. 2011). The immunological function of the spleen in fighting off infection results in changes in its structure and morphology (Bjørgen and Koppang 2021). Spleen enlargement as an adaptative response to immune activation has been observed across fish species, including damselfish (Lefebvre et al. 2004; Seppänen et al. 2009; Binning et al. 2018). We did not find evidence for spleen enlargement in the present study. This absence suggests that the doses of endotoxins may not have been sufficient to trigger an immune response, or that the timing of sampling following injection (7 days post-injection) was not appropriate to capture differences in SSI among treatments. Interestingly, the SSI values measured in the fish injected with saline and 50mg kg^{-1} in the present study were similar to those obtained for fish in the same treatments in Binning et al. (2018). Notably, the fish used here and in Binning et al. (2018) were from the same population and collected at the same time of year as in the present study. Additionally, we had a much smaller sample size per treatment (for both saline and LPS 50mg kg^{-1} injection $n = 6$ in the present study vs. $n = 16$ in Binning et al. (2018)). These factors may explain the lack of clear differences observed in SSI between our groups vs. those found in Binning et al. (2018). Spleen enlargement alone may not be an accurate measure to characterize the occurrence of an immune response given the large among-individual variation that exists among treatments (Petitjean et al. 2021)

We found altered immune gene expression in fish injected with the two highest doses of LPS (50mg kg^{-1} and 100mg kg^{-1}) and with the lowest one (3mg kg^{-1}). Responses to immune stimulants are dose and time dependent. The initial phase of the inflammatory response can be followed by either a down-regulation or an up-regulation of the immune system, which can last

449 for days (Novoa et al. 2009; Abdel-Mageid et al. 2020). A non-lethal dose of LPS can cause a
450 temporary tolerance state to LPS which causes a suppression of genes linked with the
451 inflammatory immune response (Foster and Medzhitov 2009). Doses closer to a lethal
452 concentration can cause a hyperactive innate immune state to prevent an endotoxin shock
453 caused by the accumulation of LPS within the organism (Novoa et al. 2009). In our study, the
454 intermediate doses of LPS (5mg kg^{-1}, 10mg kg^{-1}, 30mg kg^{-1}) may have caused a tolerant state,
455 which might explain the absence of immune gene alteration. Conversely, the highest doses,
456 which might be closer to lethal concentration, may have caused a hyperactive innate immune
457 state. The mechanism behind the significant alteration of immune gene expression observed at
458 the lowest dose of LPS (3mg kg^{-1}) could result from a cross-immunity response to waterborne
459 pathogenic agents. Over a lifetime, individuals can be exposed to the same pathogen multiple
460 times. During the first encounter, immune cells specialized for fighting this pathogen are
461 created and stored to provide immunity in the event of another encounter later in life. This
462 acquired immunity gives subsequent protection against this pathogen and, through cross-
463 immunity, can equally protect against other similar types of pathogens (Schmid-Hempel 2003;
464 Swennes et al. 2007; Ardia et al. 2011). Previous studies observed that repeated exposure to
465 extremely low doses of LPS through feeding enhanced the immune response of grey mullet,
466 (*Mugil cephalus* (Linnaeus, 1758) and striped catfish, (*Pangasianodon hypophthalmus*
467 Sauvage, 1878) (Bich Hang et al. 2016; Abdel-Mageid et al. 2020). In the present study, we
468 used wild-caught fish. *P. amboinensis* may have been exposed in the wild to endotoxin, similar
469 to LPS. Because of a cross-immunity, it is possible that the low dose of LPS caused a reaction
470 similar to grey mullet and catfish, which strongly responded to a very low dose of LPS after
471 repeated exposure.
472

Overall, our results suggest that there are some measurable energetic costs to mounting an immune response in Ambon damselfish. However, these costs vary in a dose- and time dependent manner, which can be difficult to capture when comparing average metabolic traits measurement without considering changes overtime. The higher doses of LPS caused an increase in $\dot{M}O_2$ uptake above pre-injection values in the first few hours post-injection with only a marginal effect on the available aerobic scope and no effect on the overall integrated energy use. Furthermore, both higher doses and the lowest dose resulted in altered immune gene expression. To avoid underestimating the energetic costs of mounting an immune response, it appears critical for future studies to consider measuring traits at different levels of biological organisation as well as to account for variation among individuals through time in traits such as oxygen uptake rate. Additionally, the costs associated with an immune challenge can differ depending on the time of the day, making the injection timing a critical element to accurately attribute changes in energy demands to the onset of an immune response.

Acknowledgements

We thank F. Quattrini, C. Demairé, S. Hapke and staff at the Lizard Island Research Station for field support, S. Babin, and P. Lopes for insightful discussions, and E. Pedersen for help with statistical analyses. ML thanks R. Lafitte for his support through the project.

Competing interests statement

The authors declare no competing interests.

Contribution statement

SAB, DGR, SSK and RB conceived the study. SAB, SC, DGR and JJM collected the data. ML, DGR and SSK analysed the data and produced the figures. ML, SAB, DGR and SSK wrote the manuscript. All authors contributed to the drafts and gave final approval for publication.

Funding statement

This study was supported by funding from the Fonds de Recherche du Québec Nature et Technologies (Postdoctoral Training Scholarships awarded to SAB and DGR), the Groupe de recherche interuniversitaire en limnologie (GRIL- International Collaboration Grant awarded to SAB and SSK), the Université de Neuchâtel (Subvention Égalité awarded to SAB) and the Swiss Science Foundation (grant No. FN 31003A-153067/1 awarded to RB).

Data availability statement

The data and analytical code for this study are available to reviewers at https://osf.io/xdsyn and will be shared publicly at the time of publication.

Figure captions

Figure 1. Relationship between mass-adjusted whole organism metabolic traits (a) highest metabolic rate, (b) lowest metabolic rate and (c) routine metabolic rate, two control groups (handling control (HC) and saline injection) and six different doses of LPS ranging from 3mg kg^{-1} to 100mg kg^{-1}. Each dot represents a single fish. The boxplots indicate the medians, 25^{th} and 75^{th} percentile.

Figure 2. Percentage of residual aerobic scope in *Pomacentrus amboinensis* damselfish across treatment post-injection. Residual aerobic scope is based on absolute value of $\dot{M}O_2$ for each fish expressed relative to their own aerobic scope calculated pre-injection. *Y*-axis represents the

percentage of residual aerobic scope for each fish within each treatment (a) handing control, (b) saline injection, (c) LPS3 (3mg kg^{-1}), LPS5 (5mg kg^{-1}), LPS10 (10mg kg^{-1}), LPS30 (30mg kg^{-1}), LPS50 (50mg kg^{-1}1) and LPS100 (100mg kg^{-1}). *X*-axis represents the time in hours post-injection.

Figure 3. Mean differences in area under the curve of *Pomacentrus amboinensis* damselfish among LPS treatment groups (a) Area under the curve (AUC) of the handling control group (purple), the saline control group (dark blue) and the LPS injected group with LPS3 (3mg kg^{-1}) (indigo), LPS5 (5mg kg^{-1}) (teal), LPS10 (10mg kg^{-1}) (forest green), LPS30 (30mg kg^{-1}) (harlequin green), LPS50 (50mg kg^{-1}) (mustard) and LPS100 (100mg kg^{-1}) (yellow). Vertical bars represent the standard deviation around the group means. (b) Estimation plots indicate the mean difference in AUC between treatments and control group. Black circles represent the effect sizes and the distribution of these effect obtained through nonparametric bootstrap sampling (5,000 samples) is represented by the shaded area. Vertical bars represent the 95% confidence intervals.

Figure 4. Partial effects of the relationship between changes in oxygen uptake ($\Delta\dot{M}O_2$) over time for 8 different doses of LPS injection (a-h) estimated from a generalized additive mixed-effects model (GAMM). $\Delta\dot{M}O_2$ is calculated by subtracting pre-injection $\dot{M}O_2$ from post-injection $\dot{M}O_2$ at comparable time periods pre- and post-injection. Values above the red line indicate an increase in $\dot{M}O_2$ relative to pre-injection values, and values below the red line indicate a decrease in $\dot{M}O_2$ relative to pre-injection values. The red solid line represents the model-estimated mean centered on zero for the smooth term and the shaded area denotes a 95% confidence interval. The vertical dotted line represents the separation between nighttime 1 to

543 10 hours after insertion in the respirometer (left side of the line) and daytime 10 to 20 hours
544 after insertion in the respirometer (right side of the line).
545
546 Figure 5. Mean differences in the spleen somatic index of *Pomacentrus amboinensis* damselfish
547 among LPS treatment groups (a) Spleen somatic index (SSI) of the handling control group
548 (purple), the saline control group (dark blue) and the LPS injected group with LPS3 (3mg kg^{-1})
549 (indigo), LPS5 (5mg kg^{-1}) (teal), LPS10 (10mg kg^{-1}) (forest green), LPS30 (30mg kg^{-1})
550 (harlequin green), LPS50 (50mg kg^{-1}) (mustard) and LPS100 (100mg kg^{-1}) (yellow). The
551 vertical black lines indicate the standard deviation around the group means. (b) Estimation plots
552 indicate the mean difference in SSI between treatments and the control group. Black circles
553 represent the effect sizes and the distribution of these effects obtained through nonparametric
554 bootstrap sampling (5,000 samples) is represented by the shaded area. Vertical bars represent
555 the 95% confidence intervals.
556

557 Figure 6. Mean differences in the immune gene expression of *Pomacentrus amboinensis*
558 damselfish among LPS treatment groups (a) Gene expression (polydom/svep1) of the handling
559 control group (purple), the saline control group (dark blue) and the LPS injected group with
560 LPS3 (3mg kg^{-1}) (indigo), LPS5 (5mg kg^{-1}) (teal), LPS10 (10mg kg^{-1}) (forest green), LPS30
561 (30mg kg^{-1}) (harlequin green), LPS50 (50mg kg^{-1}) (mustard) and LPS100 (100mg kg^{-1})
562 (yellow). Vertical bars represent the standard deviation around the group means. (b) Estimation
563 plots indicate the mean difference in gene expression between treatments and control group.
564 Black circles represent the effect sizes and the distribution of these effect obtained through
565 nonparametric bootstrap sampling (5,000 samples) is represented by the shaded area. Vertical
566 bars represent the 95% confidence intervals.

567 **References**

568

569 Abdel-Mageid, A.D., Zaki, A.G., El Senosi, Y.A., Fahmy, H.A., El Asely, A.M., Abo-Al-Ela,
570 H.G. and El-Kassas, S., 2020. Modulatory effect of lipopolysaccharide on immune-related gene
571 expression and serum protein fractionation in grey mullet, *Mugil cephalus*. Aquaculture
572 Research, **51**(4), pp.1643-1652.https://doi.org/10.1111/are.14510

573

574 Ackerman, P.A., Iwama, G.K., Thornton, J.C., 2000. Physiological and immunological effects
575 of adjuvanted *aeromonas salmonicida* vaccines on juvenile rainbow trout. J. Aqua. Animal
576 Health 12: 157–164 https://doi.org/10.1577/15488667(200006)012<0157:PAIEOA>2.0.CO;2

577

578 Ardia, D.R., Parmentier, H.K., Vogel, L.A., 2011. The role of constraints and limitation in
579 driving individual variation in immune response. Funct. Ecol **25**: 61–73.
580 https://doi.org/10.1111/j.1365-2435.2010.01759.x

581

582 Bennoit, N.R., Craig, P.M., 2020. Increased metabolic rate associated with immune stimulation
583 of heat-killed *Vibrio anguillarum* at different temperatures in zebrafish (*Danio rerio*). Comp.
584 Biochem. and Physiol.B. **250**, 110489. https://doi.org/10.1016/j.cbpb.2020.110489

585

586 Berczi, I., Bertók, L., Bereznai, T., 1966. Comparative studies on the toxicity of escherichia
587 coli lipopolysaccharide endotoxin in various animal species. Can. J. Microbiol. **12**, 1070–1071.
588 https://doi.org/10.1139/m66-143

589

590 Bich Hang, B.T., Nguyen, P.T. and Kestemont, P., 2016. Oral administration of Escherichia
591 coli lipopolysaccharide enhances the immune system of striped catfish, *Pangasianodon*
592 *hypophthalmus* (Sauvage). Aquaculture research, **47**(4), pp.1299-1308.
593 https://doi.org/10.1111/are.12589
594
595 Binning, S.A., Roche, D.G., Grutter, A.S., Colosio, S., Sun, D., Miest, J., Bshary, R., 2018.
596 Cleaner wrasse indirectly affect the cognitive performance of a damselfish through
597 ectoparasite removal. Proc. R. Soc. B **285,** 20172447. https://doi.org/10.1098/rspb.2017.2447
598
599 Bjørgen, H., Koppang, E.O., 2021. Anatomy of teleost fish immune structures and organs.
600 Immunogenetics **73**, 53–63 . https://doi.org/10.1007/s00251-020-01196-0
601
602 Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B., Sorci, G., 2003.
603 Assessing the Cost of Mounting an Immune Response. Am. Nat. **161**, 367–379.
604 https://doi.org/10.1086/346134
605
606 Bonneaud, C., Wilson, R.S., Seebacher, F., 2016. Immune-challenged fish up-regulate their
607 metabolic scope to support locomotion. PLoS One **11**, e0166028.
608 https://doi.org/10.1371/journal.pone.0166028
609
610 Burness, G., Armstrong, C., Fee, T., Tilman-Schindel, E., 2010. Is there an energetic-based
611 trade-off between thermoregulation and the acute phase response in zebra finches? J. Exp. Biol.
612 **213**, 1386–1394. https://doi.org/10.1242/jeb.027011
613

614 Burton, T., Killen, S.S., Armstrong, J.D., Metcalfe, N.B., 2011. What causes intraspecific
615 variation in resting metabolic rate and what are its ecological consequences? Proc. R. Soc. B
616 **278**, 3465–3473. https://doi.org/10.1098/rspb.2011.1778
617
618 Chabot, D., Steffensen, J.F., Farrell, A.P., 2016. The determination of standard metabolic rate
619 in fishes. J. Fish Biol. **88**, 81–121. https://doi.org/10.1111/jfb.12845
620
621 Chabot, D., Koenker, R., Farrell, A. P. 2016b. The measurement of specific dynamic action in
622 fishes. J. of Fish Biol, 88(1), 152-172. https://doi.org/10.1111/jfb.12836
623
624 Claireaux, G., and Lefrançois, C., 2007. Linking environmental variability and fish
625 performance: integration through the concept of scope for activity. Proc. R. Soc. B **1487**, 2031-
626 2041. https://doi.org/10.1098/rstb.2007.2099
627
628 Clark, T.D., Sandblom, E., Jutfelt, F., 2013. Aerobic scope measurements of fishes in an era of
629 climate change: respirometry, relevance and recommendations J. Exp. Biol. **216**, 2771–2782.
630 https://doi.org/10.1242/jeb.084251
631
632 Derting, T.L., Compton, S., 2003. Immune Response, Not Immune Maintenance, Is
633 Energetically Costly in Wild White-Footed Mice (*Peromyscus leucopus*). Physiol. Biochem
634 Zool **76**, 744–752. https://doi.org/10.1086/375662
635
636 Foster, S.L. and Medzhitov, R., 2009. Gene-specific control of the TLR-induced inflammatory
637 response. Clinical immunology, **130**(1), pp.7-15. https://doi.org/10.1016/j.clim.2008.08.015
638

639 Goater, T.M., Goater, C.P., Esch, G.W., 2014. Parasitism: the diversity and ecology of animal
640 parasites. Cambridge: Cambridge University Press.
641
642 Hadfield, J.D., 2010. MCMC Methods for Multi-Response Generalized Linear Mixed Models:
643 The MCMCglmm R Package. J. Stat. Soft. **33**, 1–22. https://doi.org/10.18637/jss.v033.i02
644
645 Halberg, F., Johnson, E.A., Brown, B.W. and Bittner, J.J., 1960. Susceptibility rhythm to E.
646 coli endotoxin and bioassay. Proceedings of the Society for Experimental Biology and
647 Medicine, 103(1), pp.142-144. https://doi.org/10.3181/00379727-103-254
648
649 Hasselquist, D., Nilsson, J.-Å., 2012. Physiological mechanisms mediating costs of immune
650 responses: what can we learn from studies of birds? Anim. Behav **83**, 1303–1312.
651 https://doi.org/10.1016/j.anbehav.2012.03.025
652
653 Ho, J., Tumkaya, T., Aryal, S., Choi, H., Claridge-Chang, A., 2019. Moving beyond P values:
654 data analysis with estimation graphics. Nat. Methods **16**, 565–566.
655 https://doi.org/10.1038/s41592-019-0470-3
656
657 Jobling, M., 1981. The influences of feeding on the metabolic rate of fishes: a short review. J.
658 Fish Biol, **18**(4), pp.385-400.https://doi.org/10.1111/j.1095-8649.1981.tb03780.x
659
660 Killen, S.S., Christensen, E.A., Cortese, D., Zavorka, L., Norin, T., Cotgrove, L., Crespel, A.,
661 Munson, A., Nati, J.J., Papatheodoulou, M. and McKenzie, D.J., 2021. Guidelines for reporting
662 methods to estimate metabolic rates by aquatic intermittent-flow respirometry. J. Exp.
663 Biol, **224**(18), jeb242522. https://doi.org/10.1242/jeb.242522

664

665 Killen, S.S., Norin, T., Halsey, L.G. 2017. Do method and species lifestyle affect measures of
666 maximum metabolic rate in fish? J. Fish Biol **90**, 1037–1046. https://doi.org/10.1111/jfb.13195

667

668 Lefebvre, F., Mounaix, B., Poizat, G., Crivelli, A.J., 2004. Impacts of the swimbladder
669 nematode *Anguillicola crassus* on *Anguilla anguilla*: variations in liver and spleen masses. J.
670 Fish Biol **64**, 435–447. https://doi.org/10.1111/j.0022-1112.2004.00309.x

671

672 Lind, C. M., Agugliaro, J., and Farrell, T. M., 2020. The metabolic response to an immune
673 challenge in a viviparous snake, *Sistrurus miliarius*. J. of Exp Biol, **223**(10), jeb225185.
674 https://doi.org/10.1242/jeb.225185

675

676 Lopes, P.C., French, S.S., Woodhams, D.C., Binning, S.A., 2021. Sickness behaviors across
677 vertebrate taxa: proximate and ultimate mechanisms. J. Exp. Biol. **224**, jeb225847.
678 https://doi.org/10.1242/jeb.225847

679

680 MacKenzie, S., Montserrat, N., Mas, M., Acerete, L., Tort, L., Krasnov, A., Goetz, F.W.,
681 Planas, J.V., 2006. Bacterial lipopolysaccharide induces apoptosis in the trout ovary. Reprod.
682 Biol. Endocrinol. 4, 46. https://doi.org/10.1186/1477-7827-4-46

683

684 Magnadottir, B., 2010. Immunological Control of Fish Diseases. Marine Biotech **12**, 361–379.
685 https://doi.org/10.1007/s10126-010-9279-x

686

687 Martin II, L.B., Han, P., Lewittes, J., Kuhlman, J.R., Klasing, K.C., Wikelski, M., 2006.
688 Phytohemagglutinin-induced skin swelling in birds: histological support for a classic

689 immunoecological technique. Funct. Ecol. **20**, 290–299. https://doi.org/10.1111/j.1365-
690 2435.2006.01094.x
691
692 Martin II, L.B., Weil, Z.M., Nelson, R.J., 2007. Immune defense and reproductive pace of life
693 in peromyscus mice. Ecology **88**, 2516–2528. https://doi.org/10.1890/07-0060.1
694
695 McKenzie, D.J., Belão, T.C., Killen, S.S., Rantin, F.T., 2015. To boldly gulp: standard
696 metabolic rate and boldness have context-dependent influences on risk-taking to breathe air in
697 a catfish. J. Exp. Biol. **218**, 3762–3770. https://doi.org/10.1242/jeb.122903
698
699 Melhado, G., Herrera M., L.G., da Cruz-Neto, A.P., 2020. Bats respond to simulated bacterial
700 infection during the active phase by reducing food intake. J. Exp. Zoo. **333**, 536–542.
701 https://doi.org/10.1002/jez.2399
702
703 Møller, A.P., Erritzøe, J., 2002. Coevolution of host immune defence and parasite-induced
704 mortality: relative spleen size and mortality in altricial birds. Oikos **99**, 95–100.
705 https://doi.org/10.1034/j.1600-0706.2002.990110.x
706
707 Mebius, R., Kraal, G., 2005. Structure and function of the spleen. Nat Rev Immunol **5**, 606–
708 616. https://doi.org/10.1038/nri1669
709
710 Methling, C., Douda, K. and Reichard, M., 2019. Intensity-dependent energetic costs in a
711 reciprocal parasitic relationship. Oecologia, 191, pp.285-294. https://doi.org/10.1007/s00442-
712 019-04504-y
713

714 Mills, S.C., Grapputo, A., Jokinen, I., Koskela, E., Mappes, T. and Poikonen, T., 2010, fitness
715 trade-offs mediated by immunosuppression costs in a small mammal. Evolution, **64**: 166-
716 179. https://doi.org/10.1111/j.1558-5646.2009.00820.x
717
718 Norin, T., Clark, T.D., 2016. Measurement and relevance of maximum metabolic rate in fishes.
719 J. Fish Biol. **88**, 122–151. https://doi.org/10.1111/jfb.12796
720
721 Novoa, B., Bowman, T.V., Zon, L. and Figueras, A., 2009. LPS response and tolerance in the
722 zebrafish (*Danio rerio*). Fish and shellfish immunology, **26**(2), pp.326-331.
723 https://doi.org/10.1016/j.fsi.2008.12.004
724
725 Otálora-Ardila, A., Herrera M, L.G., Flores-Martínez, J.J. and Welch Jr, K.C., 2016. Metabolic
726 cost of the activation of immune response in the fish-eating myotis (*Myotis vivesi*): the effects
727 of inflammation and the acute phase response. PLoS One, 11(10), p.e0164938.
728 https://doi.org/10.1371/journal.pone.0164938
729
730 Petitjean, Q., Jacquin, L., LeHénaff, M., Perrault, A., Cousseau, M., Laffaille, P., Jean, S., 2021.
731 Dose- and time-dependent effects of an immune challenge on fish across biological levels. J.
732 Exp. Zool. **335**, 250–264. https://doi.org/10.1002/jez.2430
733
734 Poulin, R., Morand, S., 2000. The Diversity of Parasites. The Quarterly Review of Biology **75**,
735 277–293. https://doi.org/10.1086/393500
736

Powell, M.D., Speare, D.J., Daley, J. and Lovy, J., 2005. Differences in metabolic response to Loma salmonae infection in juvenile rainbow trout Oncorhynchus mykiss and brook trout Salvelinus fontinalis. Diseases of aquatic organisms, 67(3), pp.233-237. https://doi.org/10.3354/dao067233

Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. Immunol. Letters **148**, 23–33. https://doi.org/10.1016/j.imlet.2012.08.003

Reyes-Cerpa, S., Masey, K., Reyes-López, F., Toro-Ascuy, D., Sandino, A.M., Imarai, M., 2012. New Advances and Contributions to Fish Biology. InTech. https://doi.org/10.5772/53504

Roche, D.G., Binning, S.A., Bosiger, Y., Johansen, J.L., Rummer, J.L., 2013. Finding the best estimates of metabolic rates in a coral reef fish. J. Exp. Biol. **216**, 2103–2110. https://doi.org/10.1242/jeb.082925

Rodgers, G. G., Tenzing, P., and Clark, T. D. 2016. Experimental methods in aquatic respirometry: the importance of mixing devices and accounting for background respiration. J. Fish Biol, **88**(1), 65-80. https://doi.org/10.1111/jfb.12848

Rosewarne, P.J., Wilson, J.M., Svendsen, J.C., 2016. Measuring maximum and standard metabolic rates using intermittent-flow respirometry: a student laboratory investigation of aerobic metabolic scope and environmental hypoxia in aquatic breathers. J. Fish Biol. **88**, 265–283. https://doi.org/10.1111/jfb.12795

762 Seppänen, E., Kuukka, H., Voutilainen, A., Huuskonen, H. and Peuhkuri, N., 2009. Metabolic
763 depression and spleen and liver enlargement in juvenile Arctic charr *Salvelinus alpinus* exposed
764 to chronic parasite infection. J. Fish Biol, **74**(3), pp.553-561.https://doi.org/10.1111/j.1095-
765 8649.2008.02144.x
766
767 Scheiermann, C., Kunisaki, Y. and Frenette, P.S., 2013. Circadian control of the immune
768 system. Nature Reviews Immunology, **13**(3), pp.190-198. https://doi.org/10.1038/nri3386
769
770 Schmid-Hempel, P., 2003. Variation in immune defence as a question of evolutionary ecology.
771 Proc. R. Soc. B **270**, 357–366. https://doi.org/10.1098/rspb.2002.2265
772
773 Sheldon, B.C., Verhulst, S., 1996. Ecological immunology: costly parasite defences and trade-
774 offs in evolutionary ecology. Trends Ecol. Evol. **11**, 317–321. https://doi.org/10.1016/0169-
775 5347(96)10039-2
776
777 Smith, G.D., Neuman-Lee, L.A., Webb, A.C., Angilletta, M.J., DeNardo, D.F., French, S.S.,
778 2017. Metabolic responses to different immune challenges and varying resource availability in
779 the side-blotched lizard (*Uta stansburiana*). J. Comp. Physiol. B. **187**, 1173–1182.
780 https://doi.org/10.1007/s00360-017-1095-4
781
782 Stahlschmidt, Z. R., and Glass, J. R., 2020. Life history and immune challenge influence
783 metabolic plasticity to food availability and acclimation temperature. Physiological and
784 Biochemical Zoology, **93**(4), 271-281. https://doi.org/10.1086/709587
785

786 Svendsen, J.C., Genz, J., Anderson, W.G., Stol, J.A., Watkinson, D.A., Enders, E.C., 2014.
787 Evidence of circadian rhythm, oxygen regulation capacity, metabolic repeatability and positive
788 correlations between forced and spontaneous maximal metabolic rates in lake sturgeon
789 *Acipenser fulvescens*. PLoS One **9**, e94693. https://doi.org/10.1371/journal.pone.0094693
790
791 Svendsen, M. B. S., Bushnell, P. G., and Steffensen, J. F., 2016. Design and setup of
792 intermittent-flow respirometry system for aquatic organisms. J. Exp. Biol, 88(1), pp. 26-50.
793 https://doi.org/10.1111/jfb.12797
794
795 Swennes, A.G., Findly, R.C., Dickerson, H.W., 2007. Cross-immunity and antibody responses
796 to different immobilisation serotypes of *Ichthyophthirius multifiliis*. Fish Shellfish Immunol **22**,
797 589–597. https://doi.org/10.1016/j.fsi.2006.07.005
798
799 Uller, T., Isaksson, C., and Olsson, M., 2006. Immune challenge reduces reproductive output
800 and growth in a lizard. Funct. Eco, 873-879. https://doi.org/10.1111/j.1365-2435.2006.01163.x
801
802 Uribe, C., Folch, H., Enríquez, R., Moran, G. J. V. M., 2011. Innate and adaptive immunity in
803 teleost fish: a review. Veterinarni medicina, **56** (10), 486. https://doi.org/10.17221/3294-
804 VETMED
805
806 Wood, S. and Wood, M.S., 2015. Package 'mgcv'. R package version, 1(29), p.729.
807
808 Wood, S.N., 2017. Generalized Additive Models: An introduction with R, 2nd ed. Chapman
809 and Hall/CRC. https://doi.org/10.1201/9781315370279
810

811 Zanuzzo, F.S., Urbinati, E.C., Nash, G.W., Gamperl, A.K., 2015. Steelhead trout *Oncorhynchus*
812 *mykiss* metabolic rate is affected by dietary Aloe vera inclusion but not by mounting an immune
813 response against formalin-killed *Aeromonas salmonicida*. J. Fish Biol. **87**, 43–53.
814 https://doi.org/10.1111/jfb.12690
815
816

Table 1 – Parameter estimates for $\Delta\dot{M}O_2$ through time and doses of LPS; 3mg kg^{-1} (LPS3), 5 mg kg^{-1} (LPS5), 10mg kg^{-1} (LPS10), 30mg kg^{-1} (LPS30), 50mg kg^{-1} (LPS50) and 100mg kg^{-1} (LPS100) using a generalized additive mixed model with fish ID as a random effect. Time is smoothed across treatment to detect specific trend overtime for each treatment. Effective degrees of freedom (edf) estimated residual degrees of freedom (ref.df) and Chi.sq are given for smoothed terms. *P* values indicate whether the estimated function (s) reject or not the null hypothesis (i.e., a flat constant function). Estimate, standard error (SE), Chi.sq and *p*-values are given for the parametric variable treatment.

Variable	**edf**	**Ref.df**	**Chi.sq**	***p*-values**
s (Time: handling control)	12.30	29	8022.4	0.005302**
s (Time: saline)	21.82	29	25067.8	0.000943***
s (Time: LPS3)	23.22	29	18760.7	0.003492**
s (Time: LPS5)	18,19	29	7679.0	0.044357*
s (Time: LPS10)	16.44	29	10785.7	0.009976**
s (Time: LPS30)	11.03	29	7472.0	0.003179**
s (Time: LPS50)	14.82	29	562.3	0.536626
s (Time: LPS100)	16.70	29	357.0	0.654444

Table 2 – Spleen somatic index (SSI) expressed in percentage, immune gene expression (polydom/svep1) and area under the curve (AUC) post-injection expressed in $\dot{M}O_2$ (mg O_2 h^{-1}) of damselfish for saline injection and across the 6 doses of LPS compared to the handling control group.

	Saline vs. handling control (**Δ** [95% CI])	LPS3 vs. handling control (**Δ** [95% CI])	LPS5 vs. handling control (**Δ** [95% CI])	LPS10 vs. handling control (**Δ** [95% CI])	LPS30 vs. handling control (**Δ** [95% CI])	LPS50 vs. handling control (**Δ** [95% CI])	LPS100 vs. handling control (**Δ** [95% CI])
Spleen somatic index (SSI)	-0.005 [0.025 – 0.006]	0.009 [0.016 – 0.027]	-0.006 [0.025 – 0.004]	0.005 [0.016 – 0.022]	0.006 [0.014 – 0.022]	-0.0002 [0.019 – 0.012]	0.021 [0.0009 – 0.0373]
Gene expression (polydom/svep1)	0.254 [-0.583 – 1.26]	2.21 [0.553 – 4.19]	0.159 [-0.859 – 1.43]	0.158 [-0.690 – 1.31]	1.18 [-0.031 – 3.07]	1.04 [0.217 – 2.1]	1.26 [0.476 – 2.32]
Area under the curve (AUC)	51.6 [-339 – 401]	80.3 [-151 – 208]	66.5 [-169 – 213]	33.8 [-199 – 166]	-71.6 [-321 – 79.3]	226 [-32.9 – 495]	63.2 [-191 – 214]

SSI, gene expression and area under the curve data values are mean differences between handling control and treatment groups with 95% bootstraped confidence interval (Δ [95% CI]).

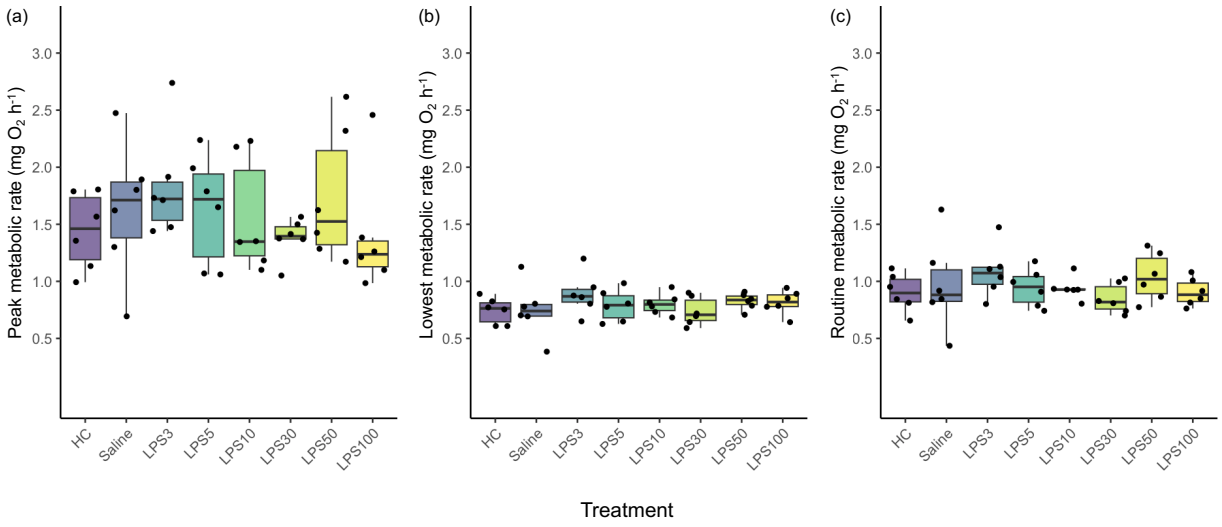

Figure 1

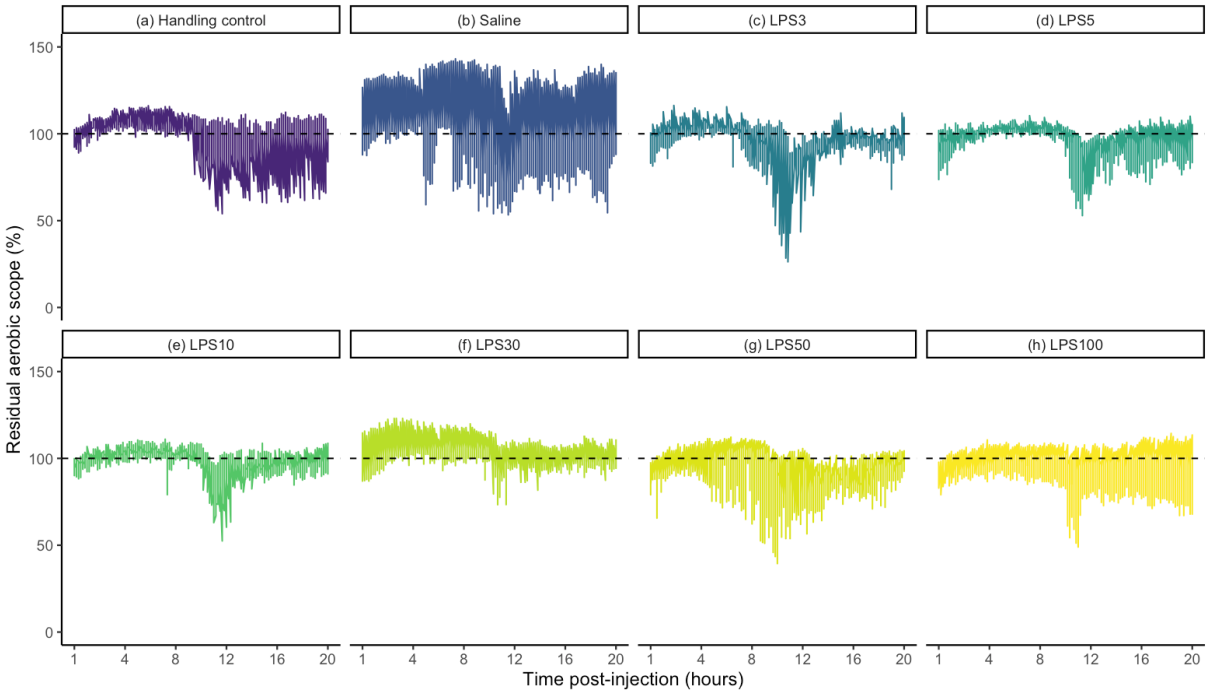

Figure 2

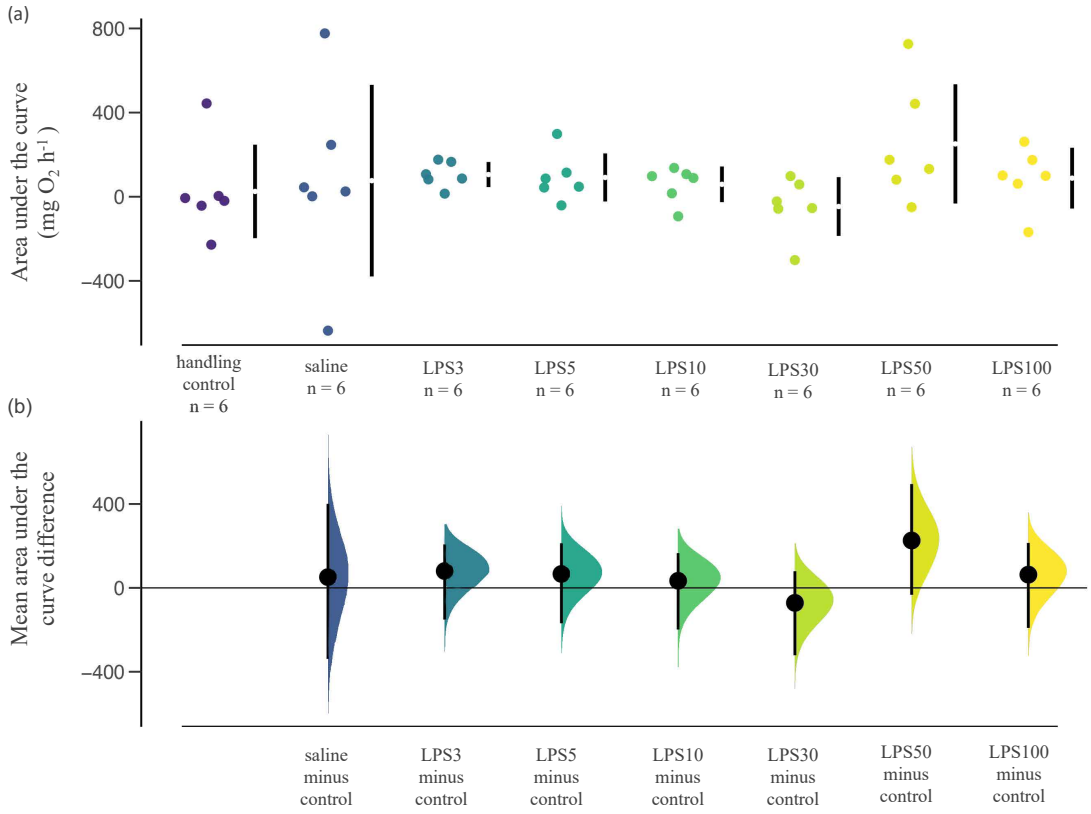

Figure 3

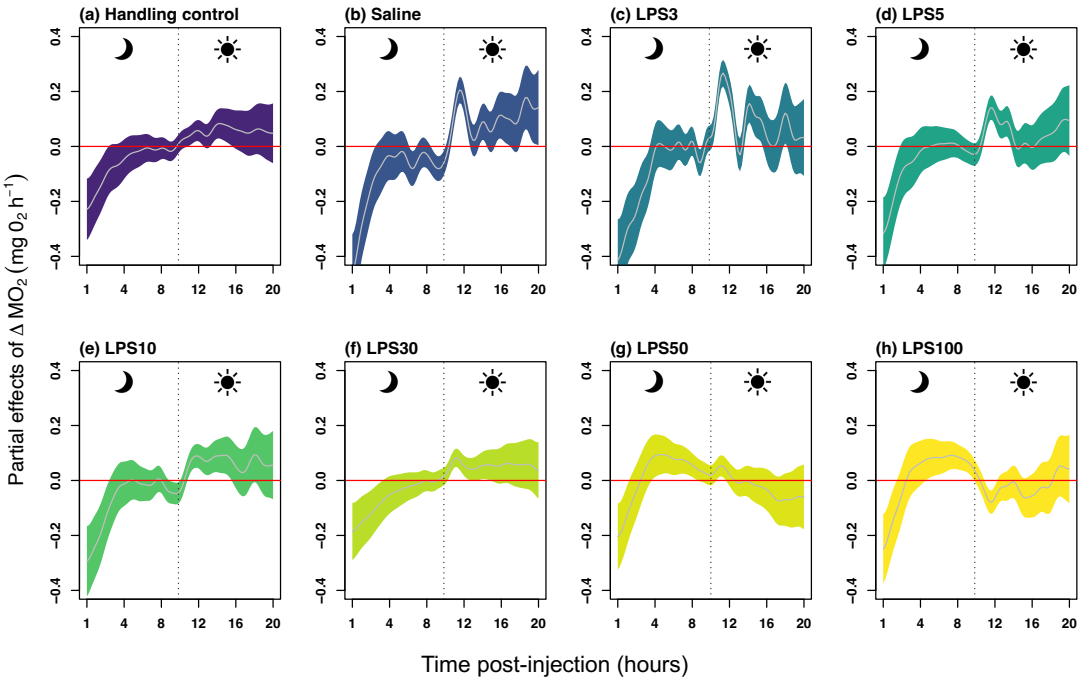

Figure 4

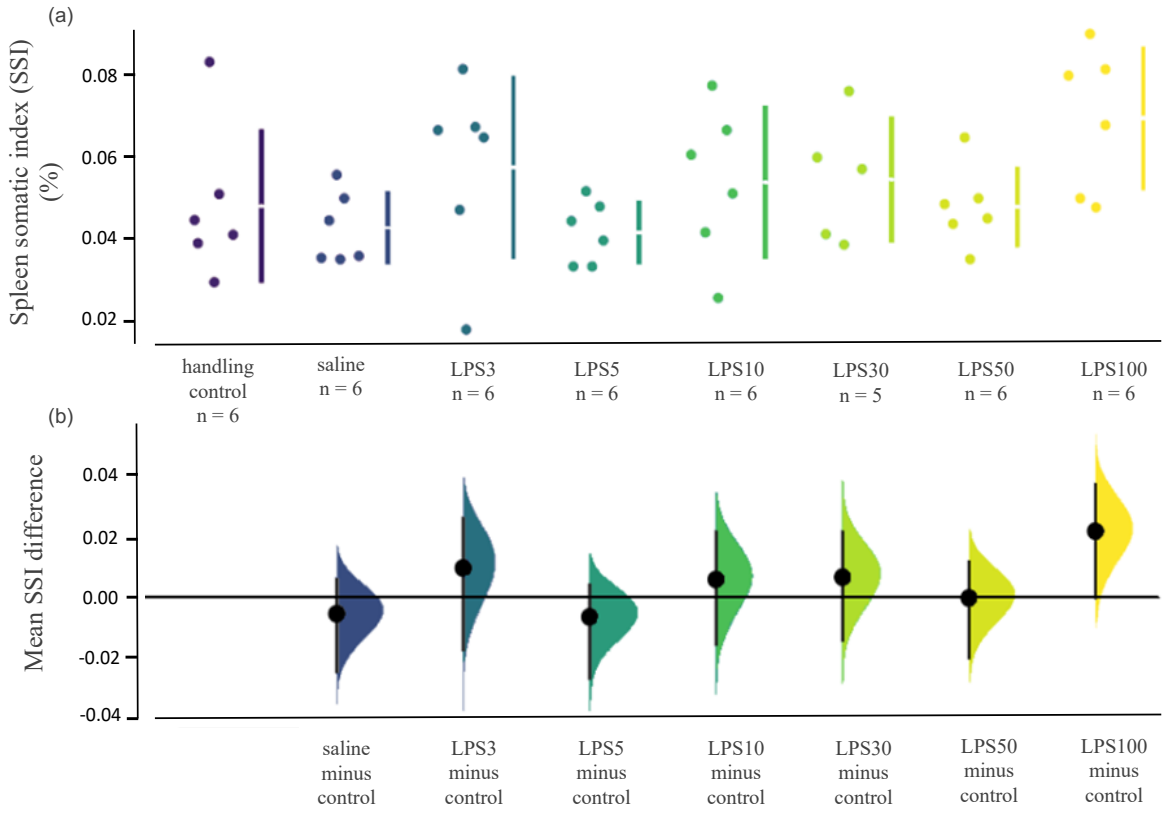

Figure 5

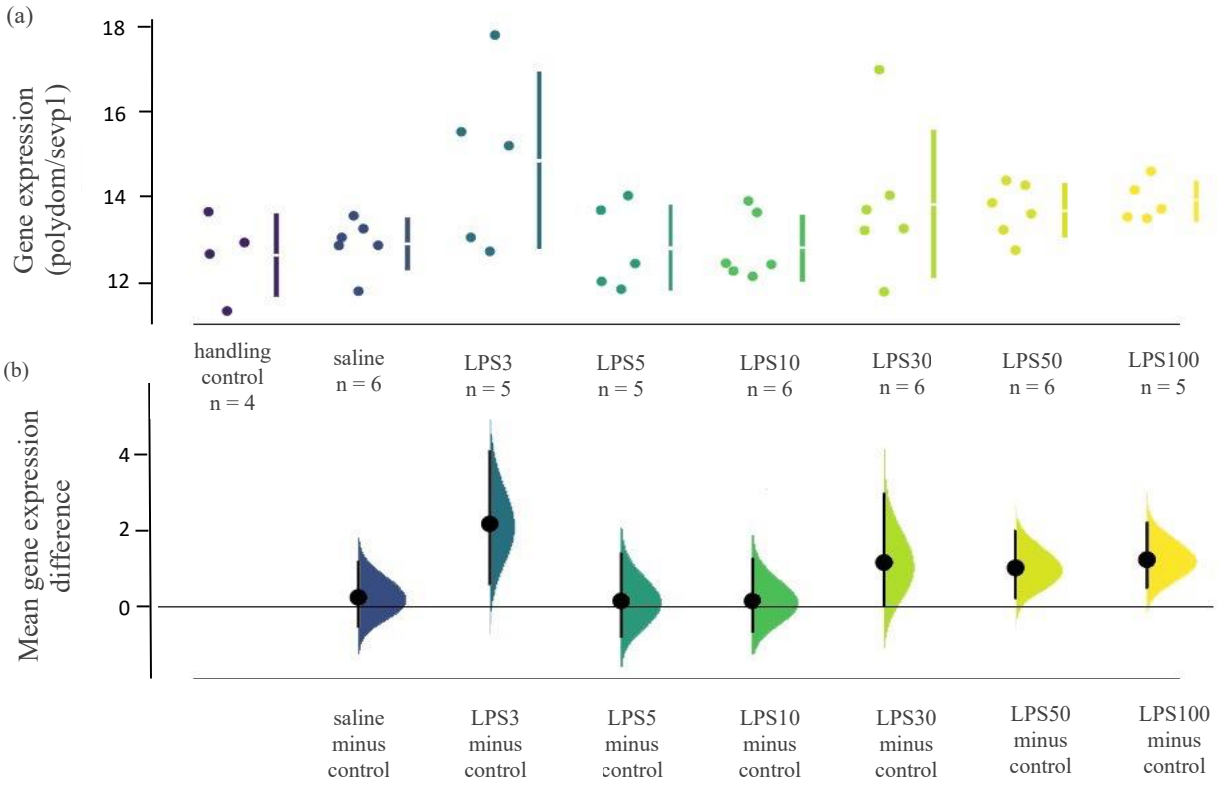

Figure 6