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

2　***amboinensis*)**

3

4　Marie Levet[1], Dominique G. Roche[2, 5], Shaun S. Killen [1,3], Simona Colosio[2], Redouan

5　Bshary[2], Joanna J. Miest[4, 6], Sandra A. Binning[1,2]

6

7　[1] Groupe de recherche interuniversitaire en limnologie (GRIL), Département de sciences

8　biologiques, Université de Montréal,1375 Av ; Thérèse-Lavoie-Roux, Montréal, QC, Canada,

9　H2V 0B3

10

11　[2] Institut de Biologie, Éco-éthologie, Université de Neuchâtel, Neuchâtel 2000, Switzerland

12

13　[3] School of Biodiversity, One Health and Veterinary Medicine,Graham Kerr Building,

14　University of Glasgow, Glasgow, Scotland, United Kingdom, G12 8QQ

15

16　[4] School of Science, University of Greenwich, Chatham Maritime, Kent, United Kingdom ME4

17　4TB

18

19　[5] Department of Biology and Institute of Environmental and Interdisciplinary Sciences,

20　Carleton University, Ottawa, ON, Canada, K1S 5B6

21

22　[6] Natural and Applied Sciences, Canterbury Christ Church University, Canterbury, Kent,

23　United Kingdom, CT1 1QU

24

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

26 **Abstract**

27 While immune responses can be energetically costly, quantifying these costs is challenging. We

28 tested the metabolic costs of immune activation in damselfish (*Pomacentrus amboinensis*

29 Bleeker, 1868) following a mass-adjusted injection of lipopolysaccharide (LPS) endotoxin.

30 Fish were divided into eight treatments: two controls (handling and saline injection) and six

31 LPS groups with concentrations ranging from 3 to 100mg kg^{-1}. We used intermittent flow

32 respirometry to measure differences in oxygen uptake (delta$\dot{M}O_2$) 20h before versus 20h after

33 LPS injection and changes in metabolic traits (lowest, routine and peak metabolic rates) as

34 proxies of the aerobic costs of metabolism. Spleen somatic index (SSI) and gene expression in

35 spleens were measured to assess immune activation. We found no difference in metabolic traits

36 or SSI but observed different non-linear patterns of delta$\dot{M}O_2$ in fish exposed to 50 and 100mg

37 kg^{-1} LPS compared to lower doses and controls. Fish exposed to high doses of LPS also had

38 lower residual aerobic scope compared to controls and lower LPS doses. Fish exposed to doses

39 of 3, 50, and 100mg kg^{-1} showed altered gene expression compared to the handling control.

40 Overall, our results suggest that immune activation has measurable effects on metabolic traits

41 that are both dose and time-dependent.

42

46

Introduction

48 Infection with macroparasites such as helminths and arthropods and/or microparasites such as

49 bacteria, protozoa, fungi or viruses can be costly for hosts and decreases host fitness (Poulin

50 and Morand, 2000; Schimdt-Hempel 2003; Goater et al. 2014). In animals, infection is often

51 linked to the activation of the host's immune system. Upon recognition of a disease-causing

52 agent, the host's innate immune system, which is responsible for non-specific immune

53 responses, activated along with a systemic inflammatory response and activation of the

54 hypothalamic-pituitary-adrenal axis; together, these form part of the acute phase response

55 (APR) to infection (Magnadottír 2010). The APR triggers a series of cellular reactions including

56 hormone and cytokine secretion; activation of lymphocyte differentiation (B-cells) and

57 proliferation (B cells and T-cells) as well as secretion of plasma protein in the liver (Møller and

58 Erritzoe 2002; Reyes-Cerpa et al. 2012) resulting in an increase in metabolic demands. For

59 example, mosquitofish (*Gambusio holbrooki* Girard, 1959) can experience a significant

60 reduction in body mass while expressing an immune response, suggesting the use of energy

61 reserves to sustain an increase in metabolic demands due to immune activation (Bonneaud et

62 al. 2016). The metabolic costs of immune activation can also be quantified by estimating

63 changes in aerobic metabolism (Powell et al. 2005; Methling et al. 2019). For instance,

64 immune-challenged zebra finches (*Taeniopygia guttata* Vieillot, 1817) increase their resting

65 metabolic rate in response to lipopolysaccharide (LPS) injection (Burness et al. 2010). These

66 results suggest a trade-off between energy allocation to immune function and other fitness-

67 enhancing activities (Sheldon and Verhulst 1996; Mills et al. 2010).

68

69 Despite some evidence for this trade-off, several studies fail to demonstrate an energetic cost

70 associated with host immune activation. For example, side-blotched lizard (*Uta stransburiana*

71 Baird and Girard, 1852), and wild white-footed mice (*Peromyscus leucopus* (Rafinesque, 1818)

72 showed no changes in their metabolic rates in the days, after being exposed to an immune

73 stimulant (Derting and Compton 2003; Smith et al.2017).

74 Several non-mutually exclusive factors could explain the inconsistencies in the energetic costs

75 of mounting an immune response observed among studies and species. For example the

76 methods used to experimentally induce non-pathogenic immune reactions in hosts vary among

77 studies. Studies commonly use phytohaemagglutinin (PHA) or LPS to trigger a host immune

78 response. Yet, each method yields differences in terms of the immune reaction it triggers.

79 Injection of PHA – a non-pathogenic antigen from a plant lectin – induces swelling (Martin et

80 al. 2006). Alternatively, LPS – an endotoxin present in gram-negative bacteria – provokes an

81 inflammatory response characterized by "sickness behaviour", such as fever, lethargy, and

82 reduced physiological function, often leading to a decrease in food intake and/or reproductive

83 success (Bonneaud et al. 2003; Melhado et al. 2020; Lopes et al. 2021). As such, the energetic

84 costs of different forms of immune stimulation may differ. For example, fish-eating myotis bats

85 (*Myotis vivesi* Ménégaux, 1901), injected with 50 μL of PHA show no change in body mass or

86 routine metabolic rate in the 54 hours post-injection (Otàlora-Ardila et al. 2016). In contrast,

87 individuals injected with 50 μL of LPS experience a decrease in body mass and a drastic

88 increase in their routine metabolic rate (up by 140-185%) within 11 hours post-injection

89 (Otàlora-Ardila et al. 2016). This discrepancy suggests a higher energetic cost associated with

90 the physiological reactions induced by LPS compared with PHA.

91

92 Another factor that may contribute to discrepencies among studies examining the energetic

93 costs of the immune response is that immune responses are species-specific, regardless of the

94 immune stimulant used to induce immune activation (Berczi et al. 1966; Martin, Weil, and

95 Nelson 2007). For instance, mammals and birds are extremely sensitive to endotoxins and react

96 strongly to LPS injection (Berczì et al. 1966; Hasselquist and Nilsson 2012). For guinea pigs,

97 (*Cavia porcellus* (Linnaeus,1758)), an injection of 10mg kg^{-1} of LPS can lead to lethal

98 endotoxin shock. In contrast, some teleost fishes are known to be remarkably tolerant to LPS,

99 with doses of 50mg kg^{-1} of LPS failing to induce sickness symptoms in coho salmon

100 (*Oncorhynhus kisutch* (Walbaum,1792)) or rainbow trout (*Salmo gaidneri* (Walbaum,1792)

101 (Berczì et al. 1966). It is noteworthy that most studies exploring the physiological costs of

102 immune activation focus on birds and mammals (Hasselquist and Nelson 2012). Similar studies

103 in reptiles, amphibians or fishes remain scarce despite known differences among taxa and

104 species (Ackerman et al. 2000; Mackenzie et al. 2006; Zanuzzo et al. 2015; Bonneaud et al.

105 2016; Bennoit and Craig 2020). Finally, experiments often study the effects of a single dose, or

106 limited doses, of a given immune stimulant without the necessary controls (i.e., a handling

107 control and a saline injection control), making it difficult to understand the dose-response of

108 hosts to endotoxin administration. Individual within species variation in metabolic rates are also

109 not typically considered in study designs despite minimum metabolic rates sometimes varying

110 greatly (2-3-fold) among individuals within the same population (Burton et al. 2011). This large

111 among-individual variation in metabolic rates makes it more difficult to detect differences

112 among treatments.

113

114 Here, we examine the metabolic costs of mounting an immune response in the coral reef

115 damselfish, (*Pomacentrus amboinensis* Bleeker, 1868)*,* by measuring oxygen uptake in

116 individuals pre- and post-immune stimulation, across a range of LPS doses. We also assess

117 differences in spleen mass and gene expression across treatments to assess the effectiveness of

118 an endotoxin injection in stimulating an immune response. The spleen is a major lymphoid

119 organ in fish and plays a critical role in clearing infection with spleen size often used as an

120 indicator of an immune response in fish (Lefebvre et al. 2004; Rauta et al. 2012). *Pomacentrus*

121 *amboinensis* are well-studied in terms of their physiology and behaviour. This species was

122 previously found to mount an immune response following injection of 50mg kg^{-1} of LPS as

123 demonstrated by an alteration in gene expression associated with immune activation (Binning

124 et al. 2018). Thus, our objectives were to (1) assess whether individuals respond physiologically

125 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 $\pm$ 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

176   sealed (maximum 15 seconds). For this first measurement, the chamber remained closed for 15

177   minutes to have continuous measurements to calculate $\dot{M}O_{2max}$. Afterwards, ten minutes loops

178   with a 240s flush, 60s wait and 300s measure cycle were taken to measure $\dot{M}O_2$. This continuous

179   periodic monitoring of $\dot{M}O_2$ in the respirometer allowed estimation of individual standard

180   metabolic rate, the minimum energy required in a post-absorptive state to maintain homeostasis,

181   overnight (hereafter: SMR) (Chabot et al. 2016). We calculated SMR as the 20th percentile of

182   measures taken overnight, excluding the first five hours after transfer to the respirometer to

183   minimise the effects of stress on estimates of SMR (Chabot et al. 2016). From these measures,

184   we were able to determine individual's aerobic scope (hereafter: AS), the total amount of energy

185   available to allocate to physiological systems including the immune system, calculated as

186   MMR-SMR (Derting and Compton 2003; Claireaux and Lefrançois 2007; Chabot et al. 2016b).

187   We fasted the fish for for 24h prior to the respirometry trials to avoid increases in $\dot{M}O_2$ caused

188   by a digestive state (Jobling 1981, Chabot et al. 2016b). The body mass of each fish was

189   determined to the nearest 0.1g before the start of each trial. Two loops were run without a fish

190   in the respirometer to measure initial and final background rates of respiration. Background

191   respiration was accounted for by subtracting oxygen consumption in an empty chamber from

192   concurrent $\dot{M}O_2$ values taken in chambers containing fish (Rodgers et al. 2016). Artificial

193   lighting was turned off, and only natural lighting was available during the rest of the trial.

194

Respirometry - Immune challenge

196   After approximately 20h in the respirometer, fish were given an immune challenge to estimate

197   the metabolic costs of mounting an innate immune response. We used lipopolysaccharide (LPS;

198   Sigma-Aldrich L2880, serotype 055:B5), a component of the *E*-coli cell wall, to elicit an

199   immune response in fish. Fish were haphazardly assigned to one of eight treatments (6 fish per

200   treatment) such that the mean fish mass was similar among treatment groups: 1) handling

201 control, 2) saline injection (0.9 % saline control), 3) 3mg kg^{-1} LPS, 4) 5mg kg^{-1} LPS, 5) 10mg

202 kg^{-1} LPS, 6) 30mg kg^{-1} LPS, 7) 50mg kg^{-1} LPS, 8) 100mg kg^{-1} LPS. Fish were removed from

203 the respirometers and injected intraperitoneally (i.e. abdominal cavity) using heparinized 29

204 gauge insulin syringes (heparin concentration: 28 mg/ml^{-1}) through a plastic bag partially filled

205 with seawater to avoid stress caused by air exposure and to protect the fish from surface

206 abrasions during handling. The volume of the injection was mass-adjusted for each fish

207 resulting in a injected volume standardized across concentrations and fish. Following injection,

208 the test fish was immediately placed back in the respirometry chamber and left for a further 20h

209 to measure $\dot{M}O_2$ post-injection. These 20h of measurements post-injection were taken over the

210 same time period as the period of measurements pre-injection. With post-injection $\dot{M}O_2$ values,

211 we measured the peak, lowest, and routine metabolic rate to compare difference in metabolic

212 traits among groups. We defined the lowest metabolic rate as the 20th percentile of $\dot{M}O_2$ values

213 post-injection and calculated the routine metabolic rate as the average $\dot{M}O_2$ values post-

214 injection. We selected the peak value of $\dot{M}O_2$ as a proxy for the peak metabolic rate post-

215 injection. Furthermore, we calculated the percentage of aerobic scope available for the fish

216 across time within each treatment by subtracting $\dot{M}O_2$ values post-injection from the aerobic

217 scope calculated pre-injection. We also estimated the total integrated post-injection oxygen

218 uptake for each individual by calculating the area under the curve (AUC) of $\dot{M}O_2$ versus time

219 post-injection. AUC was calculated as integral of $\dot{M}O_2$ post-injection using the trapezoid rule

220 (Chabot et al. 2016b). A positive AUC suggests an overall increase in energy demands

221 following injection, whereas a negative AUC suggests a decrease in energy demands following

222 injection. Post-respirometry experiments, the fish were returned to their holding tanks for a

223 week and fed twice per day.

224

225 **Tissue collection**

226 To ensure sufficient time for the systemic and the adaptive immune response to establish a

227 seven day period was observed between the LPS injection and the tissue collection. This time

228 period is sufficient for changes in gene expression to be detected in this species (Binning et al.

229 2018). Fish were euthanized in an overdose of AQUI-S® solution (AQUI-S New-Zeland Ltd).

230 Fish weight and length were measured again. The spleen was then dissected out and weighed.

231 Spleen tissue was preserved in RNAlater® (R0901 Sigma-Aldrich), frozen, and stored at -20°C

232 until they were shipped to the University of Greenwich for PCR analysis.

233 **Spleen tissue and gene expression analysis**

234 The spleen somatic index (SSI) was calculated as the proportion of the spleen in relation to the

235 total fish wet mass. The protocol used for immune gene analysis is described in Binning et al.

236 (2018). Briefly, RNA was extracted from the spleen with ReliaPrep^{TM} RNA Cell Miniprep

237 System (Promega). RNA concentration was determined using a nanodrop 2000 (Thermo

238 Scientific). The samples were then normalized to a common concentration of 50ng μl^{-1} using

239 nuclease-free water (Promega). 425 ng RNA were treated with DNAse (Quanta PerfeCta DNase

240 I Kit (QuantaBio)) and 212 ng of the treated RNA were transcribed using Quanta qScript cDNA

241 Synthesis Kit (QuantaBio) with a total volume of 10 μl following manufacturer instructions. A

242 1:10 dilution with Nuclease-free water (Promega) was performed and samples were stored at

243 4°C overnight. Gene expression analysis was performed the next day. Immunological response

244 to LPS challenge was quantified using gene polydom/svep1 (Binning et al. 2018). To ensure

245 specificity melting curves of the qPCR products were checked between 60 and 90°C with 1°C

246 intervals. Gene expression data (ct-values) were normalised against the house keeping gene

247 12s, to give dct. One fish in the LPS30 was not included in the SSI and gene expression analysis

248 due to an inability to retrieve the spleen. Two fish in the handling control, one fish in the saline

249 group, one fish exposed to 3mg kg^{-1} of LPS, one fish exposed to 5mg kg^{-1} of LPS, and one fish

250 exposed to 100mg kg^{-1} of LPS had a spleen too small to perform RNA extraction preventing

251 the analysis of immune gene expression.

252

253 **Statistical analysis**

254 All statistical analyses and data visualization were performed using R v.4.0.3 (R Core Team,

255 2016). We examined the relationship between metabolic traits (lowest, routine, and peak

256 metabolic rates) and treatment with a linear model (LM). The lowest, routine, and peak

257 metabolic rates were used as response variables, with treatment, fish mass and their interaction

258 as predictors. This allowed us to assess group differences in metabolic traits while accounting

259 for mass as a covariate. Body mass and metabolic rates were both $\log_{10}$ -transformed to

260 normalize the data. Percentage of residual aerobic scope available at each time step was

261 calculated for each fish by estimating individual AS pre-injection, from which we subtracted

262 the AS estimated post-injection. Then, we compared the average available aerobic scope among

263 treatments. Values above 100% indicate that oxygen uptake post-injection was lower than pre-

264 injection whilst values below 100% indicate an increase in oxygen uptake post-injection. To

265 observe changes in the $\dot{M}O_2$ patterns among treatments through time, we measured the

266 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

267 from the $\dot{M}O_2$ post-injection taken at the exact same time. For example, fish $\dot{M}O_2$ value taken

268 pre-injection at 7:00:00 pm were subtracted to its $\dot{M}O_2$ value taken post-injection at 7:00:00 pm

269 the following day. By doing so, we aimed to determine the change in an individual's oxygen

270 uptake relative to its own baseline over time. The first hour of $\dot{M}O_2$ measurement pre- and post-

271 injection were excluded from $\dot{M}O_2$ measurement to exclude any change in oxygen uptake linked

272 to either, the MMR measurement pre-injection or the handling/injection post-injection (Fig.S1).

273 To capture the nonlinear pattern observed for changes in $\dot{M}O_2$ over time among treatments, we

274 used a generalized additive mixed model (GAMM; function "gam" in R package "mgcv", Wood

275   and Wood 2015). GAMMs are an extension of generalized linear mixed models using smooth

276   terms alongside parametric terms. Smooth terms are a set of non-linear functions (i.e., basis

277   functions), which are multiplied each by a coefficient estimated from the data and summed.

278   Using the gam.check () function, we determined the number of basis functions needed for the

279   smooth term "time" in our model. The k parameter (i.e., number of basis functions) was set to

280   30 basis functions for the smooth term "time". Our model contained the parametric terms

281   "delta$\dot{M}O_2$" (response variable), "treatment" (explanatory variable), "mass" (explanatory

282   variable), and the smooth term "time" (explanatory variable) representing patterns related to the

283   non-linear change in delta$\dot{M}O_2$ over time (in minutes). We included the random effect 'fish ID'

284   in the model to allow smoothers to vary in intercept and slope. We used restricted maximum

285   likelihood (REML) for parameter estimation. Following diagnostic plots assessment, we used

286   the scaled t family in place of Gaussian as our data appeared heavy tailed (Wood 2017).

287   We used estimation statistics to determine differences in the spleen somatic index (SSI),

288   immune gene expression, group mean, standard deviations and treatment effect sizes with 95%

289   confidence intervals are presented using Gardner-Altman estimation plots (*R package* "

290   dabestr", Ho et al. 2019).

291   We used a Bayesian multivariate model (*R package MCMCglmm*; Hadfield et al. 2010) with a

292   Gaussian error distribution to examine covariation among three immune activation parameters:

293   SSI, immune gene expression, and the AUC. Treatment and fish mass were included in the

294   model as fixed effects. Predictor variables were mean-centered (mean = 0) and standardised to

295   one standard deviation. Visual inspection of diagnostic plots (parameters traces) indicated chain

296   convergence. All statistical analyses and data visualization were performed using R v. 4.0.3 (R

297   Core Team, 2016).

298

299   **Results**

300   *Change in metabolic traits post-injection*

301   We found no significant difference in the peak metabolic rate attained by fish across groups

302   post-injection (LM, $F_{(7,32)}$ = 0.93, p = 0.491) (Fig.1 (a)). There were also no differences in the

303   lowest metabolic rate (LM, $F_{(7,32)}$ = 1.130, p = 0.369) and routine metabolic rate (LM, $F_{(7,32)}$ =

304   0.76, p = 0.62) (Fig.1 (b-c)). The treatment-mass interaction was not significant for any of the

305   metabolic traits (peak metabolic rate: $F_{(7,32)}$ = 0.802, p = 0.591; lowest metabolic rate: $F_{(7,32)}$ =

306   1.120, p = 0.374; routine metabolic rate: $F_{(7,32)}$ = 0.605, p = 0.746). Fish mass had a significant

307   positive effect on fish routine metabolic rate ($F_{(7,32)}$ = 7.150, p = 0.011) and was marginally

308   non-significant for both the peak and lowest fish metabolic rate (repectively: $F_{(7,32)}$ = 4.053, p

309   = 0.052 ; $F_{(7,32)}$ = 4.088, p = 0.051). Fish exposed to 50mg kg^{-1} and 100mg kg^{-1} had, on average,

310   the lowest aerobic scope post-injection with, respectively, 92.57 % and 95.96% of their aerobic

311   scope available (Fig.2 (g-h). In comparison, both control groups approached nearly 100% of

312   their aerobic scope (99,30% for the handling control and 100.26% for the saline) post-injection

313   (Fig.2 (a-b)).Fish injected with doses of 30mg kg^{-1} and lower, all had greater than 95% of their

314   aerobic scope available post-injection (dose of 3mg kg^{-1} = 96.35%; dose of 5mg kg^{-1} = 96,84%;

315   dose of 10mg kg^{-1} = 98.09%; dose of 30mg kg^{-1} = 102.02%) (Fig.2 (c-f)). The peak level of

316   $\dot{M}O_2$ post injection occurred between 11 and 12h for the control groups and the LPS doses

317   ranging from 3mg kg^{-1} to 30mg kg^{-1} (Fig.2 (a-f)). For fish exposed to 50mg kg^{-1} and 100mg

318   kg^{-1}, the peak $\dot{M}O_2$ occurred between 8 and 9 h post injection (Fig.2 (g-h)). Finally, control

319   groups and LPS injected fish showed no difference in AUC (Table 2, Fig.3).

320

321   *Changes in oxygen uptake overtime*

322   In our GAMM model, the estimated degrees of freedom ranged from 12.30 to 23.22 suggesting

323   a highly nonlinear relationship between the oxygen uptake through time and LPS doses (Table

324   1). Delta$\dot{M}O_2$ tended to vary through time in fish in both control groups, and in the LPS-

325 injection treatment groups with dose of 3, 5, 10, and 30mg kg^{-1} (all p < 0.05; Table 1, Fig.4

326 (a-f)) but not in LPS treatment groups with doses of 50, and 100mg kg^{-1} (LPS50: $p = 0.53$ and

327 LPS100: $p = 0.65$; Table 1, Fig.4 (g-h)). In other words, there were changes in $\dot{M}O_2$ uptake in

328 fish exposed to 3, 5, and 10mg kg^{-1} LPS: fish in these groups initally had lower $\dot{M}O_2$ post-

329 injection compared to pre-injection, followed by oxygen uptake above pre-injection values 10

330 hours post-injection, which occurred around sunrise (time, Fig.4 ((c, e)). This pattern was also

331 observed in fish exposed to a saline injection (Fig.4 (b)). The handling control group and fish

332 injected with an LPS dose of 30mg kg^{-1} showed a similar pattern, exhibiting a steady increase

333 in $\dot{M}O_2$ over time (Fig.4 (a, f)). Interestingly, fish exposed to the two highest LPS doses (50

334 and 100mg kg^{-1}) had patterns of delta$\dot{M}O_2$ through time that did not differ from 0, suggesting a

335 relatively constant oxygen uptake both pre and post-injection.

336

337 *Changes in SSI and gene expression*

338 We found no difference in SSI between the two control groups (handling and saline injection)

339 and between the handling control (the contrast group) and the different LPS doses (Table 2,

340 Fig.5). There was no difference in immune gene expression between the handling and saline

341 control groups (Table 2, Fig.6). However, we observed a dose-dependent effect of LPS injection

342 on immune gene expression, with gene expression being altered in fish injected with the lowest

343 dose of LPS (3mg kg^{-1}) and the two highest LPS doses (50mg kg^{-1} and 100mg kg^{-1}) compared

344 to the handling control group (Table 2, Fig.6). There was also an indication of a similar pattern

345 for fish injected with 30mg kg^{-1} of LPS (mean difference in gene expression compared to the

346 handling control: 1.18 dct, 95% *CI*: -0.0031– 3.04).

347

348 *Covariation between SSI, gene expression, and metabolism*

349   We found no evidence of a strong association between SSI, gene expression, and metabolism

350   following immune stimulation (AUC–SSI r = -0.22; AUC–gene expression r = -0.26; SSI–gene

351   expression r = 0.28; Fig.S2). A more detailed examination of correlations within individual

352   treatment groups supports this finding (Fig.S3 and Fig.S4).

353

354   **Discussion**

355   Our objectives were to determine if immune stimulated coral reef fish showed difference in

356   metabolic traits and oxygen uptake over time following immune activation and if these

357   differences are dose dependent. We observed no significant difference in metabolic traits post-

358   injection across treatments. However, we found that fish exposed to LPS doses of 3mg kg^{-1},

359   5mg kg^{-1}, 10mg kg^{-1} and 30mg kg^{-1} as well as fish in both control treatments had a delta$\dot{M}O_2$

360   through time relationships that differed significantly from zero. In most cases, delta$\dot{M}O_2$ was

361   lower in the first 10 hours post-injection (night time) followed by a sharp increase in delta$\dot{M}O_2$

362   around dawn (day time), which remained elevated for the rest of the day. Fish exposed to 50mg

363   kg^{-1} and 100mg kg^{-1} LPS did not follow this trend, and instead tended to have similar delta$\dot{M}O_2$

364   values both overnight and in the daytime. Fish exposed to these doses of LPS also showed a

365   lower residual aerobic scope on average compared to both controls and lower doses of LPS.

366   We also observed altered immune gene expression in fish injected with the lowest dose (3mg

367   kg^{-1}) as well as the two highest doses (50mg kg^{-1} and 100mg kg^{-1}) compared with fish in the

368   handling control. Fish SSI did not differ across treatments. Overall, these results highlight

369   complex responses to immune system activation that depend on the dose of LPS administered.

370

371   Immune stimulation and metabolic traits

372   Our results are consistent with previous findings observed in ectotherms that showed no

373   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 ^{1} 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

473 Overall, our results suggest that there are some measurable energetic costs to mounting an

474 immune response in Ambon damselfish. However, these costs vary in a dose- and time

475 dependent manner, which can be difficult to capture when comparing average metabolic traits

476 measurement without considering changes overtime. The higher doses of LPS caused an

477 increase in $\dot{M}O_2$ uptake above pre-injection values in the first few hours post-injection with

478 only a marginal effect on the available aerobic scope and no effect on the overall integrated

479 energy use. Furthermore, both higher doses and the lowest dose resulted in altered immune gene

480 expression. To avoid underestimating the energetic costs of mounting an immune response, it

481 appears critical for future studies to consider measuring traits at different levels of biological

482 organisation as well as to account for variation among individuals through time in traits such

483 as oxygen uptake rate. Additionally, the costs associated with an immune challenge can differ

484 depending on the time of the day, making the injection timing a critical element to accurately

485 attribute changes in energy demands to the onset of an immune response.

486

487 **Acknowledgements**

488 We thank F. Quattrini, C. Demairé, S. Hapke and staff at the Lizard Island Research Station for

489 field support, S. Babin, and P. Lopes for insightful discussions, and E. Pedersen for help with

490 statistical analyses. ML thanks R. Lafitte for his support through the project.

491

492 **Competing interests statement**

493 The authors declare no competing interests.

494

495 **Contribution statement**

496   SAB, DGR, SSK and RB conceived the study. SAB, SC, DGR and JJM collected the data. ML,

497   DGR and SSK analysed the data and produced the figures. ML, SAB, DGR and SSK wrote the

498   manuscript. All authors contributed to the drafts and gave final approval for publication.

499

506   **Data availability statement**

507   The data and analytical code for this study are available to reviewers at https://osf.io/xdsyn and

508   will be shared publicly at the time of publication.

509

510

511 **References**

512

513 Abdel-Mageid, A.D., Zaki, A.G., El Senosi, Y.A., Fahmy, H.A., El Asely, A.M., Abo-Al-Ela,

514 H.G. and El-Kassas, S., 2020. Modulatory effect of lipopolysaccharide on immune-related gene

515 expression and serum protein fractionation in grey mullet, *Mugil cephalus*. Aquaculture

516 Research, **51**(4), pp.1643-1652.https://doi.org/10.1111/are.14510

517

518 Ackerman, P.A., Iwama, G.K., Thornton, J.C., 2000. Physiological and immunological effects

519 of adjuvanted *aeromonas salmonicida* vaccines on juvenile rainbow trout. J. Aqua. Animal

520 Health 12: 157–164 https://doi.org/10.1577/15488667(200006)012<0157:PAIEOA>2.0.CO;2

521

522 Ardia, D.R., Parmentier, H.K., Vogel, L.A., 2011. The role of constraints and limitation in

523 driving individual variation in immune response. Funct. Ecol **25**: 61–73.

524 https://doi.org/10.1111/j.1365-2435.2010.01759.x

525

526 Bennoit, N.R., Craig, P.M., 2020. Increased metabolic rate associated with immune stimulation

527 of heat-killed *Vibrio anguillarum* at different temperatures in zebrafish (*Danio rerio*). Comp.

528 Biochem. and Physiol.B. **250**, 110489. https://doi.org/10.1016/j.cbpb.2020.110489

529

530 Berczi, I., Bertók, L., Bereznai, T., 1966. Comparative studies on the toxicity of escherichia

531 coli lipopolysaccharide endotoxin in various animal species. Can. J. Microbiol. **12**, 1070–1071.

532 https://doi.org/10.1139/m66-143

533

534 Bich Hang, B.T., Nguyen, P.T. and Kestemont, P., 2016. Oral administration of Escherichia

535 coli lipopolysaccharide enhances the immune system of striped catfish, *Pangasianodon*

536 *hypophthalmus* (Sauvage). Aquaculture research, **47**(4), pp.1299-1308.

537 https://doi.org/10.1111/are.12589

538

539 Binning, S.A., Roche, D.G., Grutter, A.S., Colosio, S., Sun, D., Miest, J., Bshary, R., 2018.

540 Cleaner wrasse indirectly affect the cognitive performance of a damselfish through

541 ectoparasite removal. Proc. R. Soc. B **285,** 20172447. https://doi.org/10.1098/rspb.2017.2447

542

543 Bjørgen, H., Koppang, E.O., 2021. Anatomy of teleost fish immune structures and organs.

544 Immunogenetics **73**, 53–63 . https://doi.org/10.1007/s00251-020-01196-0

545

546 Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B., Sorci, G., 2003.

547 Assessing the Cost of Mounting an Immune Response. Am. Nat. **161**, 367–379.

548 https://doi.org/10.1086/346134

549

550 Bonneaud, C., Wilson, R.S., Seebacher, F., 2016. Immune-challenged fish up-regulate their

551 metabolic scope to support locomotion. PLoS One **11**, e0166028.

552 https://doi.org/10.1371/journal.pone.0166028

553

554 Burness, G., Armstrong, C., Fee, T., Tilman-Schindel, E., 2010. Is there an energetic-based

555 trade-off between thermoregulation and the acute phase response in zebra finches? J. Exp. Biol.

556 **213**, 1386–1394. https://doi.org/10.1242/jeb.027011

557

558   Burton, T., Killen, S.S., Armstrong, J.D., Metcalfe, N.B., 2011. What causes intraspecific

559   variation in resting metabolic rate and what are its ecological consequences? Proc. R. Soc. B

560   **278**, 3465–3473. https://doi.org/10.1098/rspb.2011.1778

561

562   Chabot, D., Steffensen, J.F., Farrell, A.P., 2016. The determination of standard metabolic rate

563   in fishes. J. Fish Biol. **88**, 81–121. https://doi.org/10.1111/jfb.12845

564

565   Chabot, D., Koenker, R., Farrell, A. P. 2016b. The measurement of specific dynamic action in

566   fishes. J. of Fish Biol, 88(1), 152-172. https://doi.org/10.1111/jfb.12836

567

568   Claireaux, G., and Lefrançois, C., 2007. Linking environmental variability and fish

569   performance: integration through the concept of scope for activity. Proc. R. Soc. B **1487**, 2031-

570   2041. https://doi.org/10.1098/rstb.2007.2099

571

572   Clark, T.D., Sandblom, E., Jutfelt, F., 2013. Aerobic scope measurements of fishes in an era of

573   climate change: respirometry, relevance and recommendations J. Exp. Biol. **216**, 2771–2782.

574   https://doi.org/10.1242/jeb.084251

575

576   Derting, T.L., Compton, S., 2003. Immune Response, Not Immune Maintenance, Is

577   Energetically Costly in Wild White-Footed Mice (*Peromyscus leucopus*). Physiol. Biochem

578   Zool **76**, 744–752. https://doi.org/10.1086/375662

579

580   Foster, S.L. and Medzhitov, R., 2009. Gene-specific control of the TLR-induced inflammatory

581   response. Clinical immunology, **130**(1), pp.7-15. https://doi.org/10.1016/j.clim.2008.08.015

582

583 Goater, T.M., Goater, C.P., Esch, G.W., 2014. Parasitism: the diversity and ecology of animal

584 parasites. Cambridge: Cambridge University Press.

585

586 Hadfield, J.D., 2010. MCMC Methods for Multi-Response Generalized Linear Mixed Models:

587 The MCMCglmm R Package. J. Stat. Soft. **33**, 1–22. https://doi.org/10.18637/jss.v033.i02

588

589 Halberg, F., Johnson, E.A., Brown, B.W. and Bittner, J.J., 1960. Susceptibility rhythm to E.

590 coli endotoxin and bioassay. Proceedings of the Society for Experimental Biology and

591 Medicine, 103(1), pp.142-144. https://doi.org/10.3181/00379727-103-254

592

593 Hasselquist, D., Nilsson, J.-Å., 2012. Physiological mechanisms mediating costs of immune

594 responses: what can we learn from studies of birds? Anim. Behav **83**, 1303–1312.

595 https://doi.org/10.1016/j.anbehav.2012.03.025

596

597 Ho, J., Tumkaya, T., Aryal, S., Choi, H., Claridge-Chang, A., 2019. Moving beyond P values:

598 data analysis with estimation graphics. Nat. Methods **16**, 565–566.

599 https://doi.org/10.1038/s41592-019-0470-3

600

601 Jobling, M., 1981. The influences of feeding on the metabolic rate of fishes: a short review. J.

602 Fish Biol, **18**(4), pp.385-400.https://doi.org/10.1111/j.1095-8649.1981.tb03780.x

603

604 Killen, S.S., Christensen, E.A., Cortese, D., Zavorka, L., Norin, T., Cotgrove, L., Crespel, A.,

605 Munson, A., Nati, J.J., Papatheodoulou, M. and McKenzie, D.J., 2021. Guidelines for reporting

606 methods to estimate metabolic rates by aquatic intermittent-flow respirometry. J. Exp.

607 Biol, **224**(18), jeb242522. https://doi.org/10.1242/jeb.242522

608

609   Killen, S.S., Norin, T., Halsey, L.G. 2017. Do method and species lifestyle affect measures of

610   maximum metabolic rate in fish? J. Fish Biol **90**, 1037–1046. https://doi.org/10.1111/jfb.13195

611

612   Lefebvre, F., Mounaix, B., Poizat, G., Crivelli, A.J., 2004. Impacts of the swimbladder

613   nematode *Anguillicola crassus* on *Anguilla anguilla*: variations in liver and spleen masses. J.

614   Fish Biol **64**, 435–447. https://doi.org/10.1111/j.0022-1112.2004.00309.x

615

616   Lind, C. M., Agugliaro, J., and Farrell, T. M., 2020. The metabolic response to an immune

617   challenge in a viviparous snake, *Sistrurus miliarius*. J. of Exp Biol, **223**(10), jeb225185.

618   https://doi.org/10.1242/jeb.225185

619

620   Lopes, P.C., French, S.S., Woodhams, D.C., Binning, S.A., 2021. Sickness behaviors across

621   vertebrate taxa: proximate and ultimate mechanisms. J. Exp. Biol. **224**, jeb225847.

622   https://doi.org/10.1242/jeb.225847

623

624   MacKenzie, S., Montserrat, N., Mas, M., Acerete, L., Tort, L., Krasnov, A., Goetz, F.W.,

625   Planas, J.V., 2006. Bacterial lipopolysaccharide induces apoptosis in the trout ovary. Reprod.

626   Biol. Endocrinol. 4, 46. https://doi.org/10.1186/1477-7827-4-46

627

628   Magnadottir, B., 2010. Immunological Control of Fish Diseases. Marine Biotech **12**, 361–379.

629   https://doi.org/10.1007/s10126-010-9279-x

630

631   Martin II, L.B., Han, P., Lewittes, J., Kuhlman, J.R., Klasing, K.C., Wikelski, M., 2006.

632   Phytohemagglutinin-induced skin swelling in birds: histological support for a classic

633   immunoecological technique. Funct. Ecol. **20**, 290–299. https://doi.org/10.1111/j.1365-
634   2435.2006.01094.x

635

636   Martin II, L.B., Weil, Z.M., Nelson, R.J., 2007. Immune defense and reproductive pace of life
637   in peromyscus mice. Ecology **88**, 2516–2528. https://doi.org/10.1890/07-0060.1

638

639   McKenzie, D.J., Belão, T.C., Killen, S.S., Rantin, F.T., 2015. To boldly gulp: standard
640   metabolic rate and boldness have context-dependent influences on risk-taking to breathe air in
641   a catfish. J. Exp. Biol. **218**, 3762–3770. https://doi.org/10.1242/jeb.122903

642

643   Melhado, G., Herrera M., L.G., da Cruz-Neto, A.P., 2020. Bats respond to simulated bacterial
644   infection during the active phase by reducing food intake. J. Exp. Zoo. **333**, 536–542.
645   https://doi.org/10.1002/jez.2399

646

647   Møller, A.P., Erritzøe, J., 2002. Coevolution of host immune defence and parasite-induced
648   mortality: relative spleen size and mortality in altricial birds. Oikos **99**, 95–100.
649   https://doi.org/10.1034/j.1600-0706.2002.990110.x

650

651   Mebius, R., Kraal, G., 2005. Structure and function of the spleen. Nat Rev Immunol **5**, 606–
652   616. https://doi.org/10.1038/nri1669

653

654   Methling, C., Douda, K. and Reichard, M., 2019. Intensity-dependent energetic costs in a
655   reciprocal parasitic relationship. Oecologia, 191, pp.285-294. https://doi.org/10.1007/s00442-
656   019-04504-y

657

658　　Mills, S.C., Grapputo, A., Jokinen, I., Koskela, E., Mappes, T. and Poikonen, T., 2010, fitness

659　　trade-offs mediated by immunosuppression costs in a small mammal. Evolution, **64**: 166-

660　　179. https://doi.org/10.1111/j.1558-5646.2009.00820.x

661

662　　Norin, T., Clark, T.D., 2016. Measurement and relevance of maximum metabolic rate in fishes.

663　　J. Fish Biol. **88**, 122–151. https://doi.org/10.1111/jfb.12796

664

665　　Novoa, B., Bowman, T.V., Zon, L. and Figueras, A., 2009. LPS response and tolerance in the

666　　zebrafish (*Danio rerio*). Fish and shellfish immunology, **26**(2), pp.326-331.

667　　https://doi.org/10.1016/j.fsi.2008.12.004

668

669　　Otálora-Ardila, A., Herrera M, L.G., Flores-Martínez, J.J. and Welch Jr, K.C., 2016. Metabolic

670　　cost of the activation of immune response in the fish-eating myotis (*Myotis vivesi*): the effects

671　　of　inflammation　and　the　acute　phase　response. PLoS　One, 11(10),　p.e0164938.

672　　https://doi.org/10.1371/journal.pone.0164938

673

674　　Petitjean, Q., Jacquin, L., LeHénaff, M., Perrault, A., Cousseau, M., Laffaille, P., Jean, S., 2021.

675　　Dose- and time-dependent effects of an immune challenge on fish across biological levels. J.

676　　Exp. Zool. **335**, 250–264. https://doi.org/10.1002/jez.2430

677

678　　Poulin, R., Morand, S., 2000. The Diversity of Parasites. The Quarterly Review of Biology **75**,

679　　277–293. https://doi.org/10.1086/393500

680

681 Powell, M.D., Speare, D.J., Daley, J. and Lovy, J., 2005. Differences in metabolic response to

682 Loma salmonae infection in juvenile rainbow trout Oncorhynchus mykiss and brook trout

683 Salvelinus fontinalis. Diseases of aquatic organisms, 67(3), pp.233-237.

684 https://doi.org/10.3354/dao067233

685

686 Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their

687 role in comparative immunity study: A model for higher organisms. Immunol. Letters **148**, 23–

688 33. https://doi.org/10.1016/j.imlet.2012.08.003

689

690 Reyes-Cerpa, S., Masey, K., Reyes-López, F., Toro-Ascuy, D., Sandino, A.M., Imarai, M.,

691 2012. New Advances and Contributions to Fish Biology. InTech. https://doi.org/10.5772/53504

692

693 Roche, D.G., Binning, S.A., Bosiger, Y., Johansen, J.L., Rummer, J.L., 2013. Finding the best

694 estimates of metabolic rates in a coral reef fish. J. Exp. Biol. **216**, 2103–2110.

695 https://doi.org/10.1242/jeb.082925

696

697 Rodgers, G. G., Tenzing, P., and Clark, T. D. 2016. Experimental methods in aquatic

698 respirometry: the importance of mixing devices and accounting for background respiration. J.

699 Fish Biol, **88**(1), 65-80. https://doi.org/10.1111/jfb.12848

700

701 Rosewarne, P.J., Wilson, J.M., Svendsen, J.C., 2016. Measuring maximum and standard

702 metabolic rates using intermittent-flow respirometry: a student laboratory investigation of

703 aerobic metabolic scope and environmental hypoxia in aquatic breathers. J. Fish Biol. **88**, 265–

704 283. https://doi.org/10.1111/jfb.12795

705

706   Seppänen, E., Kuukka, H., Voutilainen, A., Huuskonen, H. and Peuhkuri, N., 2009. Metabolic

707   depression and spleen and liver enlargement in juvenile Arctic charr *Salvelinus alpinus* exposed

708   to chronic parasite infection. J. Fish Biol, **74**(3), pp.553-561.https://doi.org/10.1111/j.1095-

709   8649.2008.02144.x

710

711   Scheiermann, C., Kunisaki, Y. and Frenette, P.S., 2013. Circadian control of the immune

712   system. Nature Reviews Immunology, **13**(3), pp.190-198. https://doi.org/10.1038/nri3386

713

714   Schmid-Hempel, P., 2003. Variation in immune defence as a question of evolutionary ecology.

715   Proc. R. Soc. B **270**, 357–366. https://doi.org/10.1098/rspb.2002.2265

716

717   Sheldon, B.C., Verhulst, S., 1996. Ecological immunology: costly parasite defences and trade-

718   offs in evolutionary ecology. Trends Ecol. Evol. **11**, 317–321. https://doi.org/10.1016/0169-

719   5347(96)10039-2

720

721   Smith, G.D., Neuman-Lee, L.A., Webb, A.C., Angilletta, M.J., DeNardo, D.F., French, S.S.,

722   2017. Metabolic responses to different immune challenges and varying resource availability in

723   the side-blotched lizard (*Uta stansburiana*). J. Comp. Physiol. B. **187**, 1173–1182.

724   https://doi.org/10.1007/s00360-017-1095-4

725

726   Stahlschmidt, Z. R., and Glass, J. R., 2020. Life history and immune challenge influence

727   metabolic plasticity to food availability and acclimation temperature. Physiological and

728   Biochemical Zoology, **93**(4), 271-281. https://doi.org/10.1086/709587

729

730   Svendsen, J.C., Genz, J., Anderson, W.G., Stol, J.A., Watkinson, D.A., Enders, E.C., 2014.

731   Evidence of circadian rhythm, oxygen regulation capacity, metabolic repeatability and positive

732   correlations between forced and spontaneous maximal metabolic rates in lake sturgeon

733   *Acipenser fulvescens*. PLoS One **9**, e94693. https://doi.org/10.1371/journal.pone.0094693

734

735   Svendsen, M. B. S., Bushnell, P. G., and Steffensen, J. F., 2016. Design and setup of

736   intermittent-flow respirometry system for aquatic organisms. J. Exp. Biol, 88(1), pp. 26-50.

737   https://doi.org/10.1111/jfb.12797

738

739   Swennes, A.G., Findly, R.C., Dickerson, H.W., 2007. Cross-immunity and antibody responses

740   to different immobilisation serotypes of *Ichthyophthirius multifiliis*. Fish Shellfish Immunol **22**,

741   589–597. https://doi.org/10.1016/j.fsi.2006.07.005

742

743   Uller, T., Isaksson, C., and Olsson, M., 2006. Immune challenge reduces reproductive output

744   and growth in a lizard. Funct. Eco, 873-879. https://doi.org/10.1111/j.1365-2435.2006.01163.x

745

746   Uribe, C., Folch, H., Enríquez, R., Moran, G. J. V. M., 2011. Innate and adaptive immunity in

747   teleost fish: a review. Veterinarni medicina, **56** (10), 486. https://doi.org/10.17221/3294-

748   VETMED

749

750   Wood, S. and Wood, M.S., 2015. Package 'mgcv'. R package version, 1(29), p.729.

751

752   Wood, S.N., 2017. Generalized Additive Models: An introduction with R, 2nd ed. Chapman

753   and Hall/CRC. https://doi.org/10.1201/9781315370279

754

755　Zanuzzo, F.S., Urbinati, E.C., Nash, G.W., Gamperl, A.K., 2015. Steelhead trout *Oncorhynchus*

756　*mykiss* metabolic rate is affected by dietary Aloe vera inclusion but not by mounting an immune

757　response against formalin-killed *Aeromonas salmonicida*. J. Fish Biol. **87**, 43–53.

758　https://doi.org/10.1111/jfb.12690

759

760

761 **Figure captions**

762

763 Figure 1. Relationship between mass-adjusted whole organism metabolic traits (a) highest

764 metabolic rate, (b) lowest metabolic rate and (c) routine metabolic rate, two control groups

765 (handling control (HC) and saline injection) and six different doses of LPS ranging from 3mg

766 kg^{-1} to 100mg kg^{-1}. Each dot represents a single fish. The boxplots indicate the medians, 25th

767 and 75th percentile.

768

769 Figure 2. Percentage of residual aerobic scope in *Pomacentrus amboinensis* damselfish across

770 treatment post-injection. Residual aerobic scope is based on absolute value of $\dot{M}O_2$ for each fish

771 expressed relative to their own aerobic scope calculated pre-injection. *Y*-axis represents the

772 percentage of residual aerobic scope for each fish within each treatment (a) handing control, (b)

773 saline injection, (c) LPS3 (3mg kg^{-1}), LPS5 (5mg kg^{-1}), LPS10 (10mg kg^{-1}), LPS30 (30mg kg^{-1})

774 $^{-1}$), LPS50 (50mg kg^{-1}1) and LPS100 (100mg kg^{-1}). *X*-axis represents the time in hours post-

775 injection.

776

777 Figure 3. Mean differences in area under the curve of *Pomacentrus amboinensis* damselfish

778 among LPS treatment groups (a) Area under the curve (AUC) of the handling control group

779 (purple), the saline control group (dark blue) and the LPS injected group with LPS3 (3mg kg^{-1})

780 (indigo), LPS5 (5mg kg^{-1}) (teal), LPS10 (10mg kg^{-1}) (forest green), LPS30 (30mg kg^{-1})

781 (harlequin green), LPS50 (50mg kg^{-1}) (mustard) and LPS100 (100mg kg^{-1}) (yellow). Vertical

782 bars represent the standard deviation around the group means. (b) Estimation plots indicate the

783 mean difference in AUC between treatments and control group. Black circles represent the

784 effect sizes and the distribution of these effect obtained through nonparametric bootstrap

785   sampling (5,000 samples) is represented by the shaded area. Vertical bars represent the 95%

786   confidence intervals.

787   Figure 4. Partial effects of the relationship between changes in oxygen uptake ($\Delta\dot{M}O_2$) over

788   time for 8 different doses of LPS injection (a-h) estimated from a generalized additive mixed-

789   effects model (GAMM). $\Delta\dot{M}O_2$ is calculated by subtracting pre-injection $\dot{M}O_2$ from post-

790   injection $\dot{M}O_2$ at comparable time periods pre- and post-injection. Values above the red line

791   indicate an increase in $\dot{M}O_2$ relative to pre-injection values, and values below the red line

792   indicate a decrease in $\dot{M}O_2$ relative to pre-injection values. The red solid line represents the

793   model-estimated mean centered on zero for the smooth term and the shaded area denotes a 95%

794   confidence interval. The vertical dotted line represents the separation between nighttime 1 to

795   10 hours after insertion in the respirometer (left side of the line) and daytime 10 to 20 hours

796   after insertion in the respirometer (right side of the line).

797

798   Figure 5. Mean differences in the spleen somatic index of *Pomacentrus amboinensis* damselfish

799   among LPS treatment groups (a) Spleen somatic index (SSI) of the handling control group

800   (purple), the saline control group (dark blue) and the LPS injected group with LPS3 (3mg kg^{-1})

801   (indigo), LPS5 (5mg kg^{-1}) (teal), LPS10 (10mg kg^{-1}) (forest green), LPS30 (30mg kg^{-1})

802   (harlequin green), LPS50 (50mg kg^{-1}) (mustard) and LPS100 (100mg kg^{-1}) (yellow). The

803   vertical black lines indicate the standard deviation around the group means. (b) Estimation plots

804   indicate the mean difference in SSI between treatments and the control group. Black circles

805   represent the effect sizes and the distribution of these effects obtained through nonparametric

806   bootstrap sampling (5,000 samples) is represented by the shaded area. Vertical bars represent

807   the 95% confidence intervals.

808

809   Figure 6. Mean differences in the immune gene expression of *Pomacentrus amboinensis*

810   damselfish among LPS treatment groups (a) Gene expression (polydom/svep1) of the handling

811   control group (purple), the saline control group (dark blue) and the LPS injected group with

812   LPS3 (3mg kg^{-1}) (indigo), LPS5 (5mg kg^{-1}) (teal), LPS10 (10mg kg^{-1}) (forest green), LPS30

813   (30mg kg^{-1}) (harlequin green), LPS50 (50mg kg^{-1}) (mustard) and LPS100 (100mg kg^{-1})

814   (yellow). Vertical bars represent the standard deviation around the group means. (b) Estimation

815   plots indicate the mean difference in gene expression between treatments and control group.

816   Black circles represent the effect sizes and the distribution of these effect obtained through

817   nonparametric bootstrap sampling (5,000 samples) is represented by the shaded area. Vertical

818   bars represent the 95% confidence intervals.

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]).
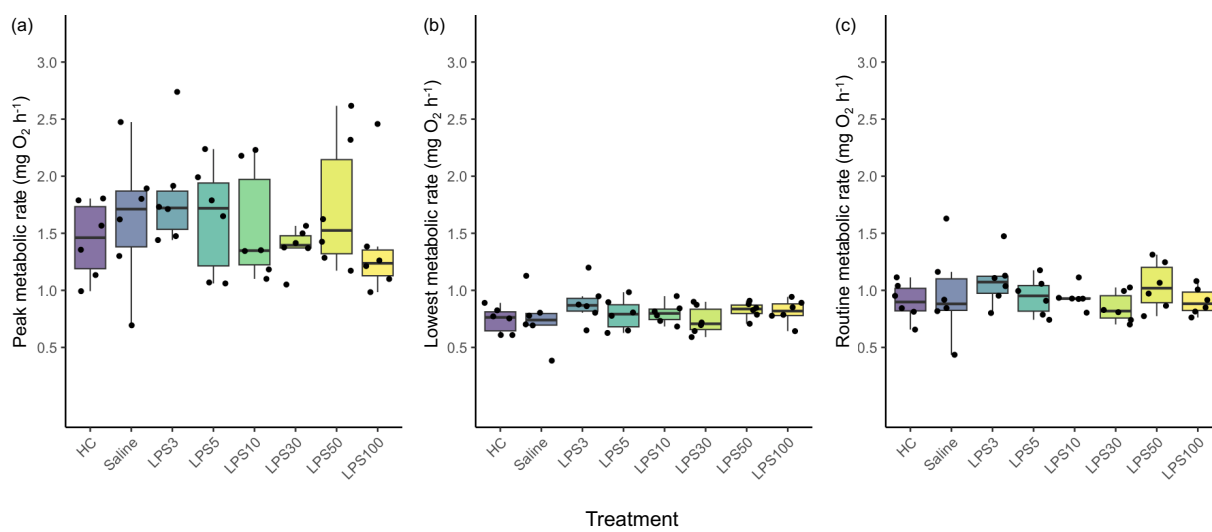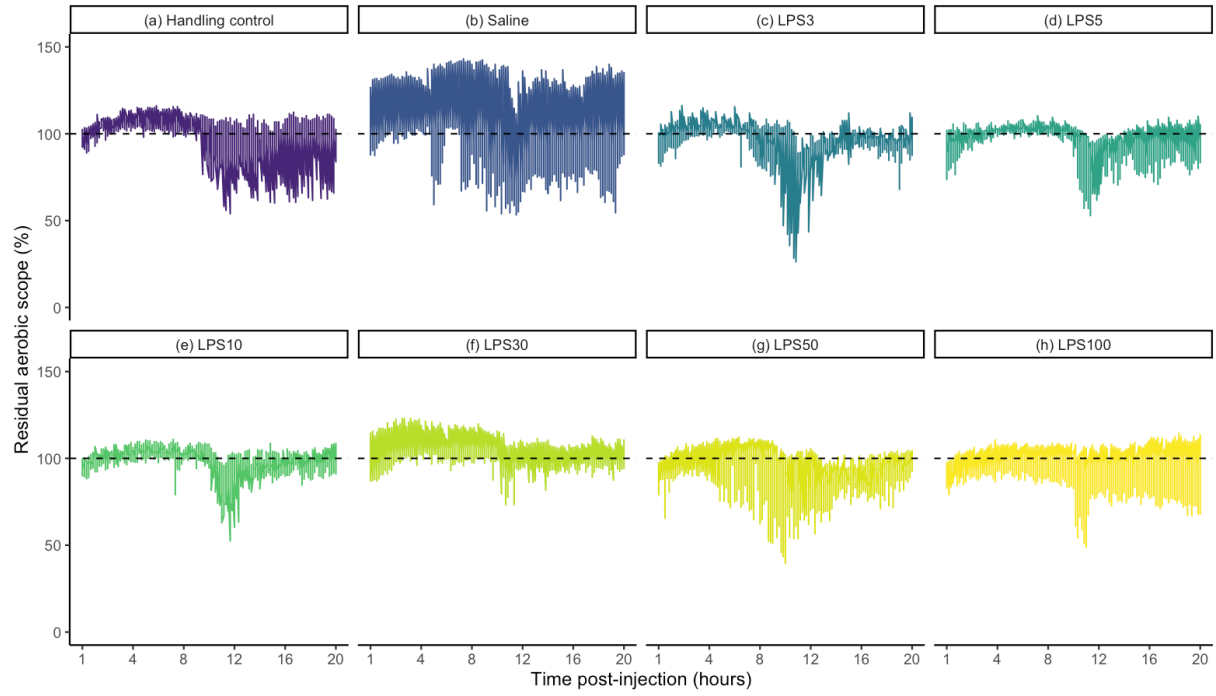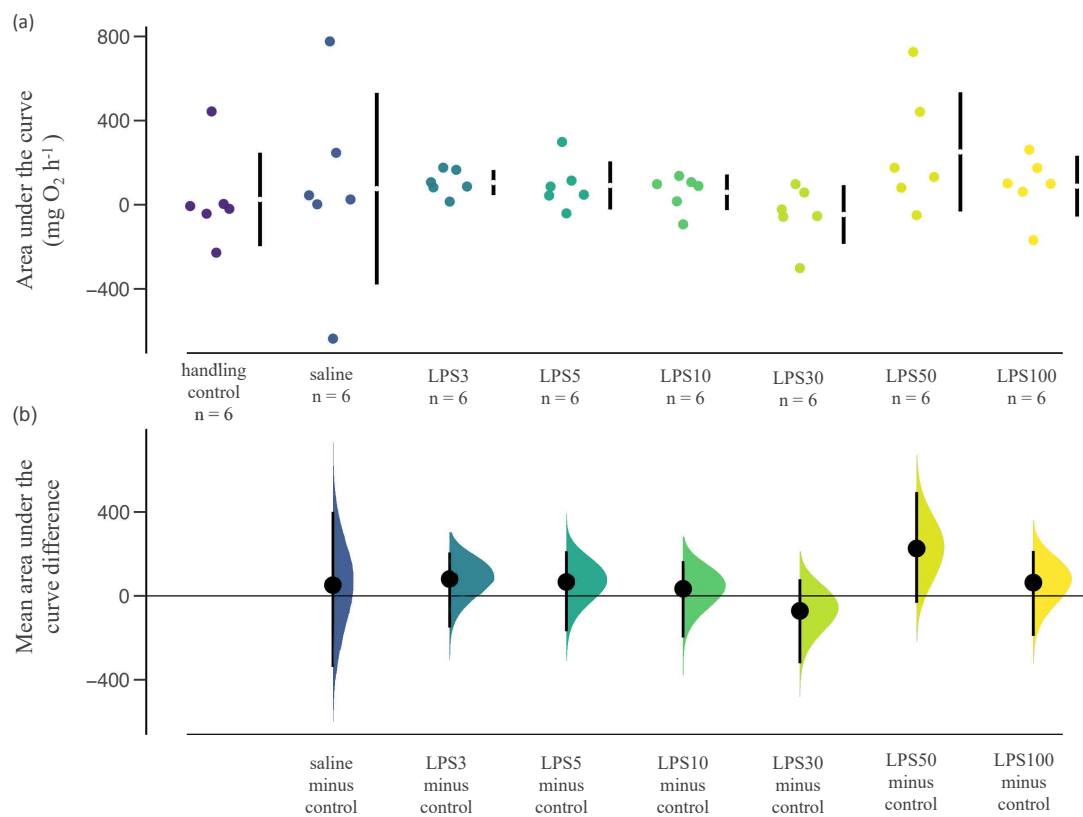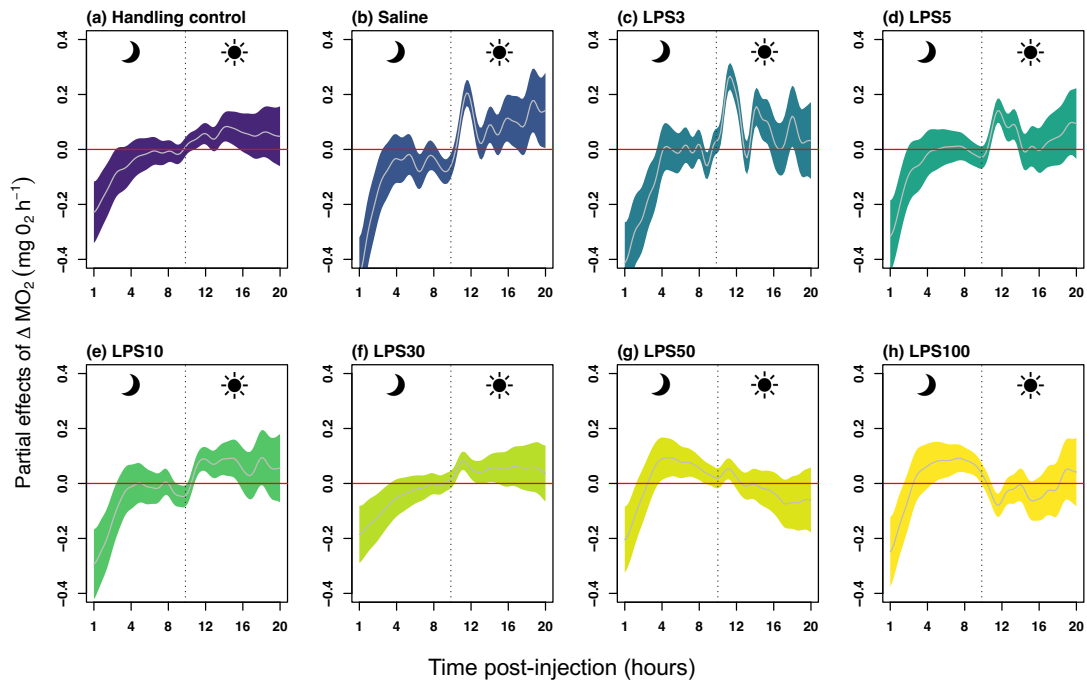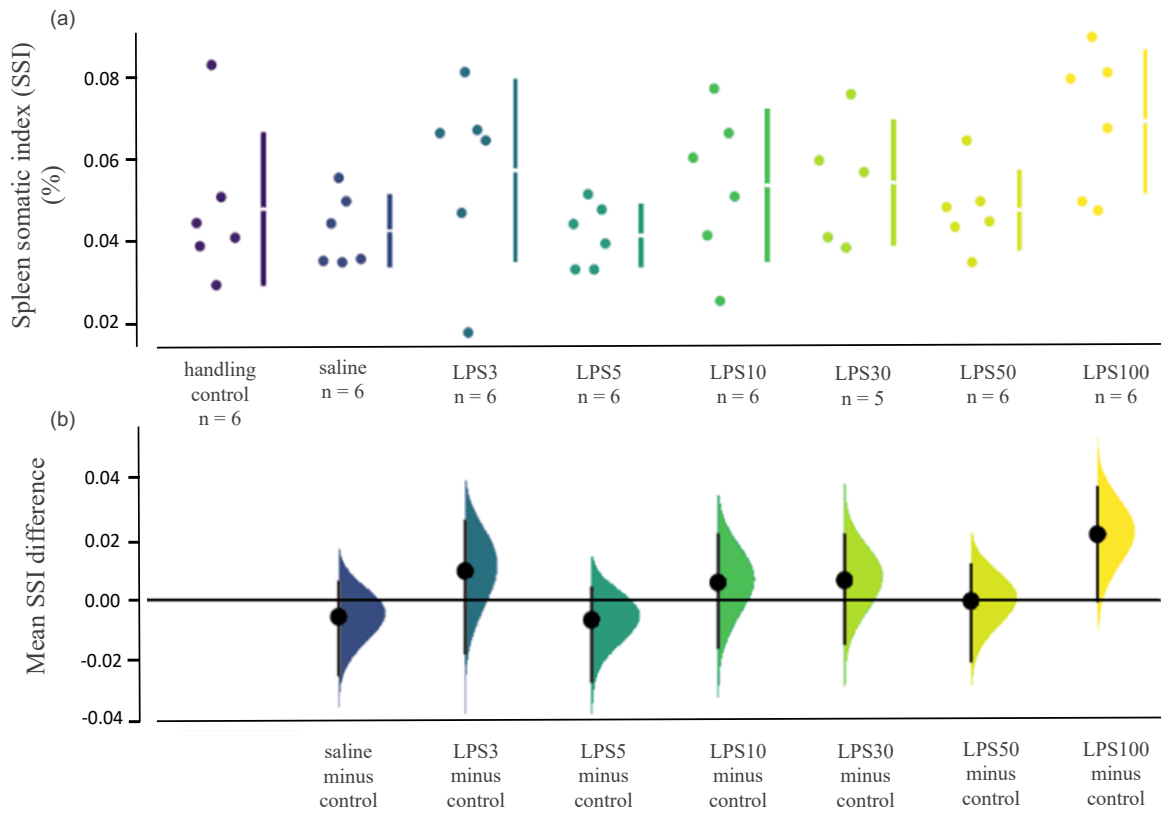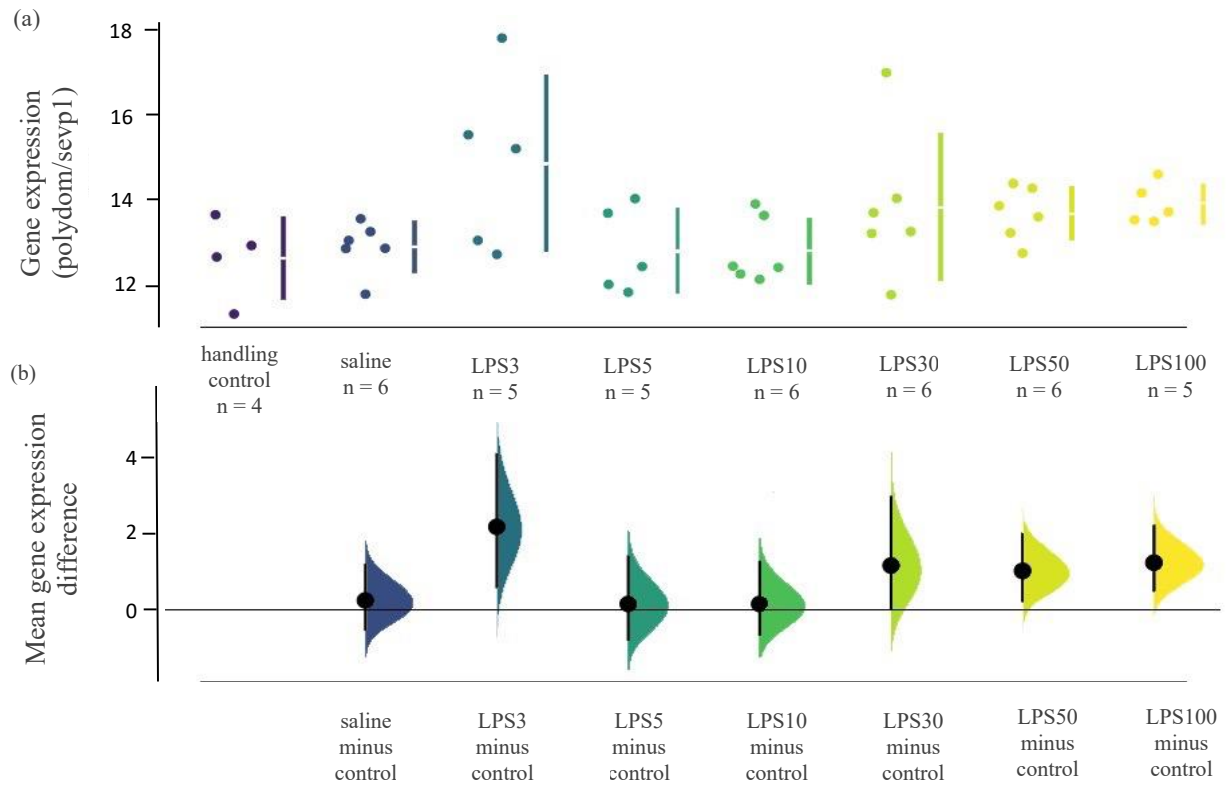
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6