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Half the story: thermal effects on within-host infectious disease progression in a warming climate

Running head: Immunity in a warming climate

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PRIMARY RESEARCH ARTICLE

29 **Abstract**

30 Immune defence is temperature-dependent in cold-blooded vertebrates (CBVs) and
31 thus directly impacted by global warming. We asked whether immunity and within-
32 host infectious disease progression are altered in CBVs under realistic climate
33 warming in a seasonal mid-latitude setting. Going further, we also asked how large
34 thermal effects are in relation to the effects of other environmental variation in such a
35 setting (critical to our ability to project infectious disease dynamics from thermal
36 relationships alone). We employed the three-spined stickleback and three
37 ecologically-relevant parasite infections as a “wild” model. To generate a realistic
38 climatic warming scenario we used naturalistic outdoors mesocosms with precise
39 temperature control. We also conducted laboratory experiments to estimate thermal
40 effects on immunity and within-host infectious disease progression under controlled
41 conditions. As experimental readouts we measured disease progression for the
42 parasites and expression in 14 immune-associated genes (providing insight into
43 immunophenotypic responses). Our mesocosm experiment demonstrated significant
44 perturbation due to modest warming (+2°C), altering the magnitude and phenology
45 of disease. Our laboratory experiments demonstrated substantial thermal effects.
46 Prevailing thermal effects were more important than lagged thermal effects and
47 disease progression increased or decreased in severity with increasing temperature
48 in an infection-specific way. Combining laboratory-determined thermal effects with
49 our mesocosm data, we used inverse modelling to partition seasonal variation in
50 *Saprolegnia* disease progression into a thermal effect and a latent
51 immunocompetence effect (driven by non-thermal environmental variation and
52 correlating with immune gene expression). The immunocompetence effect was large,
53 accounting for at least as much variation in *Saprolegnia* disease as the thermal
54 effect. This suggests that managers of CBV populations in variable environments
55 may not be able to reliably project infectious disease risk from thermal data alone.
56 Nevertheless, such projections would be improved by primarily considering
57 prevailing (not lagged) temperature variation and by incorporating validated
58 measures of individual immunocompetence.

59

60

61 **Introduction**

62 During infection, host immunity constrains the effectiveness with which a parasite
63 exploits its host, determining disease outcome. In cold-blooded animals this within-
64 host tension is modulated by environmental temperature, as both host immunity and
65 parasite development are thermally dependent (Jackson & Tinsley, 2002; Garner *et al.*,
66 2011), each with a given thermal reaction norm (Scheiner, 1993). Where these
67 reaction norms do not perfectly offset each other (Jackson & Tinsley, 2002),
68 temperature changes, such as those generated during global warming, may shift
69 susceptibility and disease progression within hosts. In turn, this may contribute to the
70 wider dynamics of disease through changing the production rate of propagules (in
71 definitive hosts) or the within-host survival of larval stages (in intermediate hosts). In
72 natural environments, the size of thermal effects, and how these measure against
73 the effects of non-thermal environmental variation (including variation driven
74 indirectly by temperature regimen), is very poorly understood. Thus, it is equally
75 poorly understood whether incremental warming would affect infectious disease
76 systems mostly directly through thermal effects or indirectly through temperature-
77 driven environmental variation. This dichotomy is key to our ability to project
78 infectious disease dynamics on the basis of thermal relationships alone.

79

80 Given the above uncertainties, we set out to measure thermal effects on immunity
81 and infectious disease progression in a cold-blooded vertebrate (CBV) model and to
82 place these effects within the context of other natural environmental effects. We
83 specifically focussed on within-host processes (excluding extra-host processes
84 contributing to transmission) and considered a seasonal mid-latitude study system,
85 which allowed the analytically powerful approach of using sinusoid functions to
86 disentangle the contributions of distinct seasonally variable drivers. We created a
87 realistic warming scenario, where we superimposed a thermal increment upon
88 natural year-round environmental cycles, and observed the resulting variation. This
89 allowed us to measure the perturbation caused by warming; but, critically, by itself
90 did not allow us to quantify the separate thermal and non-thermal processes
91 determining the observed outcomes. Crucially, we took the important further step of
92 combining infection and thermal measurements from the realistic scenario with
93 estimates from laboratory experiments where we had characterized thermal effects

94 precisely under controlled conditions. Taking a systems (inverse modelling) approach
95 we were then able to use sinusoid functions to analytically decompose the relative
96 contributions of thermal and non-thermal environmental effects.

97

98 We employed the mid-latitude three-spined stickleback (*Gasterosteus aculeatus*)
99 and its pathogens as a natural cold-blooded vertebrate (CBV) model. We kept in
100 mind that, in variable temperature regimens in natural habitats, past thermal variation
101 may feed forwards effects on physiological responses (Jackson & Tinsley, 2002;
102 Podrabsky & Somero, 2004; Raffel *et al.*, 2006, 2013, 2015; Garner *et al.*, 2011;
103 Murdock *et al.*, 2012; Dittmar *et al.*, 2014; Altman *et al.*, 2016). Our laboratory
104 experiments below therefore incorporated thermal change, allowing us to assess the
105 importance of both prevailing and time-lagged thermal effects on infectious disease
106 progression under natural seasonal thermal variation.

107

108 As phenotypic readouts we directly measured infection outcomes (Viney *et al.*, 2005)
109 in three ecologically-relevant infection systems with differing modes of established
110 infection. The directly-transmitted oomycete *Saprolegnia parasitica* (see Jiang *et al.*,
111 2013) produces a rapidly proliferating mould-like infection following initial
112 colonization by spores. Once established, these infections cause acute disease,
113 often overwhelming small fish hosts within hours or a few days post-infection. The
114 life history of the gyrodactylid monogenean *Gyrodactylus gasterostei* (see Harris,
115 1982), in contrast, is based on precocious (born near full size), directly-transmitted
116 viviparous flukes. A specialised larval transmission stage is absent: suprapopulations
117 persisting through *in situ* proliferation on individual hosts and the migration of
118 individuals from host to host. Gyrodactylid infections cause significant disease on
119 small fish that, if not fatal, may be self-limiting over a time scale of weeks or months.
120 In the cestode *Schistocephalus solidus* (see Barber & Scharsack, 2010) the
121 stickleback is the second intermediate host in an indirect life cycle, becoming
122 infected through the ingestion of copepod first intermediates. The non-proliferating *S.*
123 *solidus* plerocercoid larva may grow to great relative size (up to 50% of host weight,
124 or more), causing significant chronic disease and deformity over months or even
125 years. Our measurements for the respective infection systems (body surface
126 coverage by mycelia in *Saprolegnia*, abundance in *Gyrodactylus*, plerocercoid
127 weight in *Schistocephalus*) are in each case clear surrogates for disease

128 severity (Roberts, 2012). To provide insight into thermal effects on
129 immunocompetence we also measured (mRNA) expression for 14 immune-
130 associated genes representing different pathways (Hablützel *et al.*, 2016).

131

132 We quantified thermal effects under controlled conditions in two separate laboratory
133 experimental designs. These employed relatively large (but ecologically relevant)
134 temperature variations in order to increase the precision of estimated effects (i.e.,
135 maximizing the signal to noise ratio). One experiment examined the effects of
136 constant temperatures and of short-term temperature change, and the other the
137 effects of long-term cold exposures followed by periods of rising temperature
138 (simulating spring-like warming following winter). To generate the realistic warming
139 scenario mentioned above we conducted an outdoors mesocosm experiment using
140 an array of semi-natural tank habitats. We monitored phenotypes monthly, for a year
141 (from one autumn to the next), in a cohort of initially post-larval fish maintained in the
142 mesocosm tanks. The design was repeated twice, in separate successive years with
143 different fish cohorts. Half of the tanks were unheated and exposed to natural
144 temperature variation, whilst the other half were heated (precisely, using immersion
145 heaters with differential thermostatic control) to 2°C above the temperature of the
146 unheated tanks. This increment represents a large, but not unrealistic, stochastic
147 variation in mean temperature between successive years (O'Reilly *et al.*, 2015;
148 Sharma *et al.*, 2015) in temperate zone aquatic habitats. Such increases would be
149 expected to be more common, if as the Intergovernmental Panel on Climate Change
150 (IPCC) predicts, there is up to a 4.8°C rise in global mean surface temperature by
151 2100 (IPCC, 2014).

152

153 Our study aimed to represent processes in the field as far as possible whilst, at the
154 same time, exerting sufficient experimental control. Although, natural temperature
155 and photoperiod aside, tanks in our mesocosm experiment were not a fully natural
156 environment, they did undergo naturalistic cycles. Thus, seasonally variable
157 planktonic assemblages formed within the mesocosms and stickleback underwent
158 seasonal patterns of immune gene expression (Brown *et al.*, 2016), albeit that these
159 patterns were diminished from those seen in the wild (Hablützel *et al.*, 2016).
160 Furthermore, all of our experiments utilized quarantined anti-parasite treated wild fish
161 that had been acclimatized to laboratory or mesocosm conditions. In this choice of

162 hosts we aimed for subjects with as natural a phenotype as possible, but lacking
163 directly-transmitted pathogens capable of producing epidemics that might confound
164 the experimental structure. This approach was important given the likelihood that
165 laboratory-raised animals would have phenotypes very unrepresentative of the
166 wild (Robertson *et al.*, 2016).

167

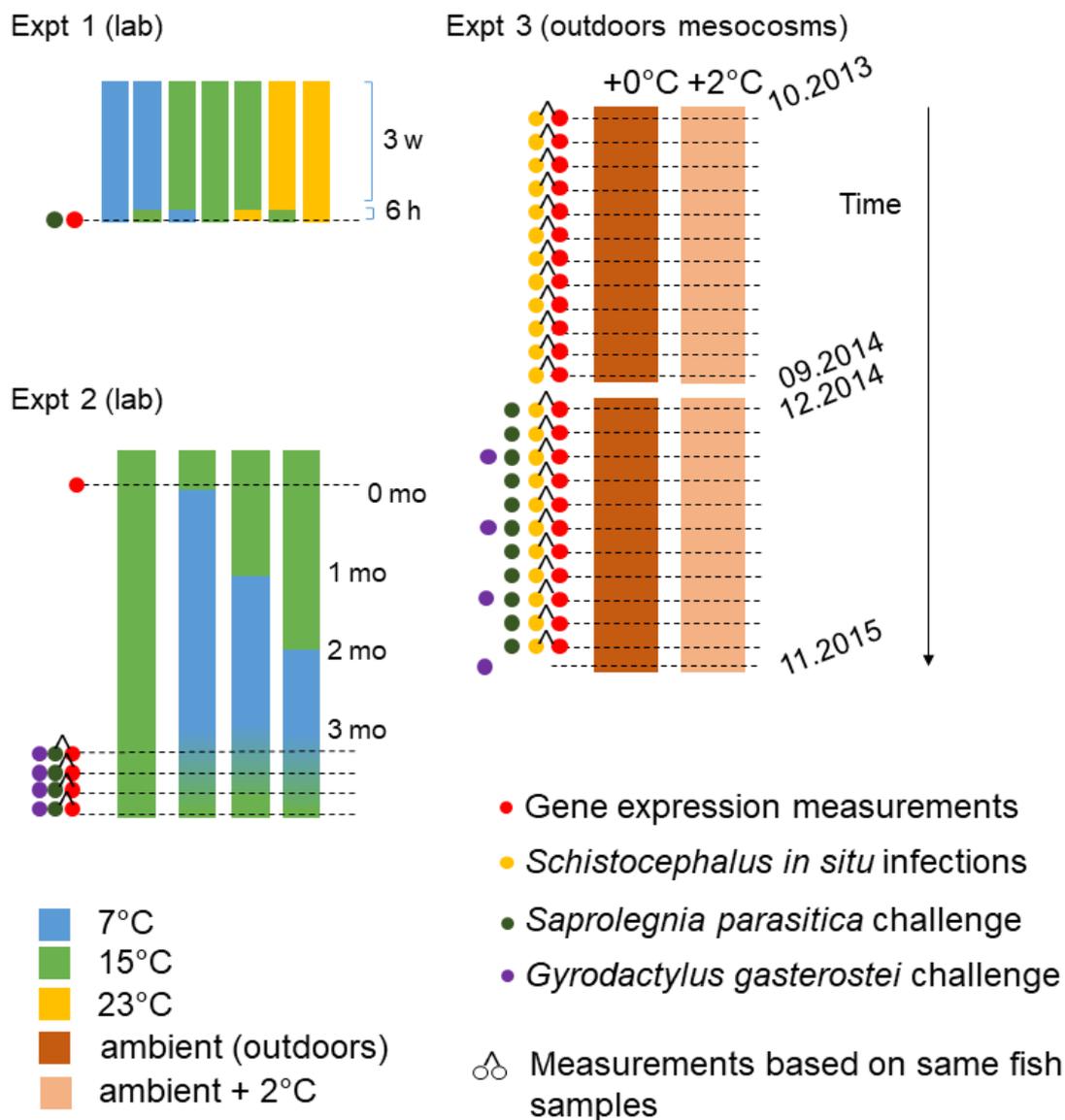
168 Below we thus ask whether immunity and infectious disease progression in a model
169 naturally-occurring CBV are detectably perturbed in a realistic, seasonal, climate
170 warming scenario. We measure the size of thermal influences in the laboratory and
171 ask whether these are mediated by prevailing and lagged effects. Finally, combining
172 the different elements of our study (as outlined above), we partition thermal effects
173 on disease progression from effects due to other temporal environmental variation
174 and ask whether thermal effects are dominant in a natural seasonal environment.

175

176 **Materials and methods**

177 *Terminology*

178 For gene expression, we define prevailing thermal effects as those due to
179 temperature around the time of measurement and lagged effects as those due to
180 temperature at some interval before the time of measurement. For infections,
181 prevailing and lagged temperature effects are defined in relation to the timing of
182 parasite invasion. Prevailing thermal effects are those due to temperature within the
183 timeframe of infection. Lagged thermal effects are those due to temperature prior to
184 infection.



185

186

187 **Fig. 1** Overview of experiments (expts) 1-3, showing timeline for temperature
 188 regimens (colour blocks), experimental time points (dotted lines) and experimental
 189 readouts associated with these points (circles). In the representation of experiment 2
 190 the timings at the end of the experiment are not shown to exact scale for simplicity
 191 (precise timings are given in the materials and methods). For *Saprolegnia* and
 192 *Gyrodactylus* challenges, the time point shown is that for initial exposure.
 193 Abbreviations: h, hours; w, weeks, mo, months. Sample sizes within cells of these
 194 experiments are given in Tables S2-S4.

195

196 *Experimental designs and methods*

197 *Overview.* We carried out two laboratory experiments to characterize thermal effects
198 on infection and immunity under controlled conditions. Both of these featured
199 factorial combinations of prevailing and lagged temperature treatments. In the first
200 experiment (experiment 1) we subjected fish to different constant temperatures and
201 then to short-term temperature shifts. In the second (experiment 2) we subjected fish
202 to differing long-term cold temperature regimens (simulating winters of different
203 length) followed by synchronized convergence on a warmer temperature (simulating
204 spring-like warming). In a third experiment (experiment 3), to simulate climate
205 warming in a naturalistic seasonal environment, we maintained fish year-round in
206 semi-natural outdoor mesocosms, superimposing a small thermal increment upon
207 natural thermal variation. The structure of these experiments (involving experimental
208 manipulations of >1500 fish) is summarised in Fig. 1 and described in detail below
209 and in Supplementary appendix S1.

210 *Experiment 1 (prevailing temperature vs short-term lagged effects in the laboratory).*

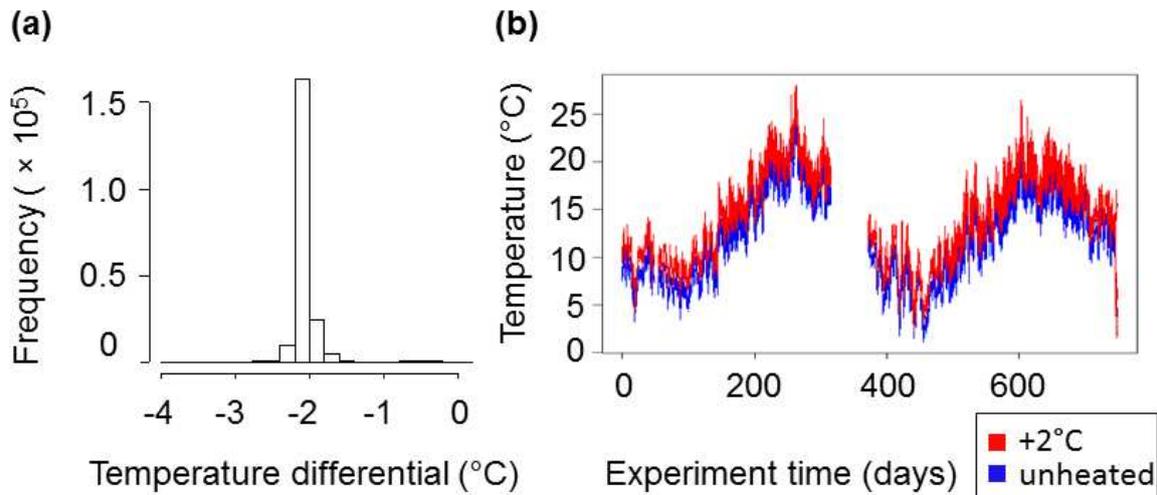
211 Wild *G. aculeatus* captured at Roath Brook, Cardiff, Wales, U.K. (RBK; 51.4998°, -
212 3.1688°) in October 2014 and 2015 were transferred to the aquarium facility at
213 Cardiff University. Here they were quarantined at a density of <1 individual L⁻¹ in 30 L
214 fresh water tanks at 15±0.5°C with 18L:6D photoperiod. All individuals were treated
215 for parasites using adaptations of treatments listed by Shinn & Bron (2012). Initially
216 fish were subjected to submersion in 0.004% formaldehyde solution for a total of 1 h
217 over a 1.5 h period (30 min exposure: 30 min rest in freshwater: 30 min exposure).
218 Following a further 24 h in fresh water, fish were then treated with praziquantel
219 (Vetark) according to the manufacturer's instructions (4 mg L⁻¹ for 48 h). Following
220 this treatment, fish were maintained for 1 week in 1% aquarium salt solution and
221 0.002 g L⁻¹ methylene blue to prevent secondary bacterial or fungal infection and
222 manually cleared of any remaining gyrodactylid infections following Schelkle *et al.*
223 (2009). Uninfected fish were then returned to fresh water (in 30L tanks, as above)
224 and acclimatised to laboratory conditions for a further one month quarantine period
225 (during which they were monitored for overt infections). Acclimatized fish were
226 weighed and measured (standardized body length, mm; body weight, mg) and
227 randomly allocated to 3 different groups (Fig. 1) that were respectively maintained at
228 7, 15 or 23°C for 3 weeks. Temperature treatment groups were then further sub-

229 divided (randomly) into temperature shift treatment groups. For the next 6 h these
230 temperature shift treatment groups were either maintained at the same temperature
231 as before (constant temperature groups), or shifted between temperatures (7 to
232 15°C, 23 to 15°C, 15 to 7°C and 15 to 23°C) (Fig. 1). Temperature treatments were
233 achieved within a suite of adjoining climate controlled rooms, in which temperature
234 varied $\pm 0.5^\circ\text{C}$ around the set temperature. After the 6 h temperature shift (lagged)
235 treatment, fish in all groups were subjected to *S. parasitica* exposure as described
236 below. Post-exposure, fish continued to be maintained at their final (prevailing)
237 temperature treatment until the sampling endpoint (72 h post-exposure). This
238 experiment was performed in eight time blocks (1-4 in 2014 and 5-8 in 2015); blocks
239 1-4 were excluded from analyses of infection outcome due to low overt symptom
240 rate. Fish from blocks 1-4 were processed for gene expression measurements.
241 Analyses of gene expression were thus based on blocks carried out in 2014 and
242 analyses of infection on blocks carried out in 2015. Final sample sizes entering
243 analyses (excluding losses due to technical failure) are broken down by experimental
244 cell in Table S2. All maintenance subsequent to the initial acclimation period and
245 before challenge exposure points was in 30 L fresh water tanks at a density of <1
246 individual L^{-1} and subject to a 18L:6D photoperiod. Fish were fed daily (*ad libitum*) on
247 chironomid larvae throughout the experiment.

248 *Experiment 2 (prevailing temperature vs long-term lagged effects in the laboratory).*
249 This experiment was carried out in two blocks separate in time: in the first of these *S.*
250 *parasitica* exposures were applied and in the other *G. gasterostei* exposures. Wild *G.*
251 *aculeatus* were captured at RBK in February 2014 (*Saprolegnia* block) and October
252 2014 (*Gyrodactylus* block). Treatment and acclimatization of fish prior to experiment
253 2 was as for experiment 1 (see above). Acclimatized fish were weighed and
254 measured (as above) and a random baseline sample preserved for gene expression
255 measurements. The remaining individuals were allocated to one of 4 long-term
256 temperature treatment (simulated winter length) groups. Over a total of 3 subsequent
257 months, these groups were first maintained at 15°C for 0, 1, 2 or 3 months and then,
258 respectively, at 7°C for 3, 2, 1 or 0 months (i.e., simulated winters of 0-3 months at
259 7°C with a synchronized end). Following this 3-month (lagged) treatment the group
260 already at 15°C continued to be maintained at this temperature, whilst those at 7°C
261 were raised to 15°C for the remainder of the experiment (Fig. 1). This 7-15°C

262 transition simulated an episode of rapid early spring warming and was carried out at
263 slightly different rates in the *Saprolegnia* and *Gyrodactylus* blocks (for operational
264 reasons). For the *Saprolegnia* block: temperature was raised at a rate of 1-2°C day⁻¹
265 over one week. For the *Gyrodactylus* block: temperature was raised at a rate of 0.5-
266 1°C day⁻¹ over two weeks. Groups of fish from each of the simulated winter length
267 groups were subject to *S. parasitica* or *G. gasterostei* exposures (as described
268 below) at the end of the long-term temperature treatment, during the warming period,
269 and following the warming period. Average temperatures (prevailing temperature
270 treatments) on exposure days for the groups starting at 7°C were either 7, 7.5, 12.5
271 or 15°C for the *Saprolegnia* block and either 7, 9.5, 13 or 15 °C for the *Gyrodactylus*
272 block. Final sample sizes entering analyses are broken down by experimental cell in
273 Table S3. Post-exposure, fish continued to be subject to the wider experimental
274 thermal regimen (acclimation to 15°C and then subsequent maintenance at 15°C)
275 until the planned sampling endpoint. Other operational conditions were as described
276 for experiment 1.

277 *Experiment 3 (+2°C thermal manipulation superimposed upon natural environmental*
278 *cycles in outdoors mesocosms)*. We utilized a system of outdoor mesocosms (12 ×
279 300 L recirculating tanks) at Aberystwyth University, U.K. equipped with precise
280 automatic temperature control and temperature monitoring. Six tanks were
281 unheated, whilst another 6 were thermostatically heated to 2.0326±0.0006°C above
282 ambient temperature (Fig. 2). Within this system we maintained separate *G.*
283 *aculeatus* year cohorts (see below) in 2013-2014 (October to September) and 2014-
284 2015 (December to November). Detailed technical specification of the recirculation,



285

286 **Fig. 2** Manipulation of temperature in mesocosm experiment (experiment 3). (a)
 287 Temperature differential between heated and unheated tanks based on 5-minutely
 288 recording (average temperature in heated tanks – average temperature in unheated
 289 tanks). (b) Temporal thermal variation in mesocosms: scatterplot of 5-minutely
 290 temperature recording for individual tanks. Experiment days are timed from
 291 November 4th 2013.

292

293 water quality management, environmental enrichment, temperature control and
 294 monitoring, stocking levels and sampling protocols are provided in Supplementary
 295 appendix S1. Briefly, fish were maintained at low biomass densities $<0.05 \text{ g L}^{-1}$. They
 296 were fed daily with standard amounts of chironomid larvae, weekly supplemented
 297 with cladocerans. A small two-level manipulation of ration, orthogonal to the main
 298 explanatory variables of interest here, was carried out (by tank, in factorial
 299 combination with temperature treatment) as part of another study and a term for
 300 ration is included in statistical analyses below. For both iterations of the experiment
 301 post-larval young-of-the-year fish were captured in the wild at Llyn Frongoch (FRN;
 302 52.3599, -3.8773), U.K., late in the breeding season, or after the end of the breeding
 303 season. To promote fish health during the subsequent experiment, all fish were
 304 subject to consecutive prophylactic anthelmintic praziquantel treatments (Hablützel
 305 *et al.*, 2016). Prior to the commencement of the experiment, fish were acclimatized
 306 for 4-6 weeks within the mesocosm system. Salinity was maintained throughout at
 307 1‰ (10 g L^{-1}) as a prophylactic measure to suppress opportunistic microbial

308 infections. Fish were sampled monthly from the mesocosm system for gene
309 expression measurements (October 2013 – September 2014; December 2014 –
310 October 2015). Ten individuals per month were taken from each thermal treatment
311 (1-2 individuals from each tank each month, in a sequence that approximately
312 equalized the number of fish taken from each tank in each quarter). These fish were
313 individually netted and immediately killed by concussion and then decerebration and
314 stored in RNA stabilization solution following Hablützel *et al.* (2016). Upon thawing
315 (prior to gene expression analysis, see below) they were dabbed dry, weighed and
316 measured (as above) and the abdominal cavity scanned for *Schistocephalus*
317 plerocercoids via a ventral incision. Total weight of any plerocercoid infection was
318 recorded and subtracted from the weight of the host. In the 2014-2015 experiment
319 run samples of fish were removed monthly (December 2014 - October 2015), for
320 exposure to *S. parasitica*, and separate samples of fish were removed quarterly
321 (February, May, August, November 2015), for exposure to *G. gasterostei*. These fish
322 were drawn in approximately equal numbers from the thermal treatments and
323 transported to Cardiff University for experimental infection procedures. Here, fish
324 were weighed and measured (as above) and maintained individually in 1L containers
325 exposed to ambient thermal variation in an outdoors facility. Salt concentration of the
326 water was reduced (from mesocosm levels) by 0.5% per day over two days, and
327 hosts were infected after a further day in fresh water (3 days after removal from the
328 mesocosm system). At Cardiff, all fish were fed daily, *ad libitum*, on chironomid
329 larvae and maintained under a single temperature regimen (outside ambient); any
330 effect of the mesocosm temperature treatment on infection outcome was thus a
331 lagged one. Final sample sizes entering analyses are broken down by experimental
332 cell in Table S4.

333 *Challenge infection protocols*

334 All experimentally challenged fish were maintained individually in standard 1 L
335 containers with 100% water changes every 48h and fed daily (*ad libitum*) on
336 chironomid larvae.

337 *Saprolegnia parasitica*. Isolate CBS223.65 of *S. parasitica*, derived in 1965 from
338 *Esox lucius* was used in challenge infections. Except in experiment 2 (see next), all
339 individual fish were subject to 30s ami-momi technique (Hatai & Hoshiai, 1993;

340 Stueland *et al.*, 2005) to increase permissiveness to infection and then either
341 exposed to $3 \times 10^5 \text{ L}^{-1}$ *S. parasitica* spore suspension for 24 h, or left non-exposed
342 but with otherwise identical maintenance conditions (control). For experiment 2 the
343 following exposure conditions were used: 1) no exposure (control); 2) ami-momi
344 treatment only; 3) exposure to *S. parasitica* spores following ami-momi treatment; 4)
345 exposure to *S. parasitica* spores without ami-momi treatment. Spore suspensions
346 prepared following Jiang *et al.* (2013) were generated independently for each
347 individual fish (or less frequently for pairs of fish) directly from a central stock of
348 CB223.65. At 72 h post-infection (p.i.) fish were individually netted and immediately
349 killed by concussion and then decerebration. (Extensive trials indicated that fish that
350 had not developed overt infection by 72 h p.i. did not subsequently develop
351 symptoms.) All specimens were rapidly weighed, measured (as above) and imaged
352 (in lateral view; digital Nikon S3600 camera) and then immediately preserved whole
353 in RNA stabilization solution (Hablützel *et al.*, 2016) for gene expression analysis.
354 Presence of *Schistocephalus* was determined via a ventral incision made to aid the
355 penetration of RNA stabilization solution (see Hablützel *et al.*, 2016). Using digital
356 images (above), the freehand selection tool in *ImageJ* (Abramoff *et al.*, 2004) was
357 employed to measure the overall surface area of the fish and the surface area
358 covered in erupted *S. parasitica* mycelia. Infection intensity was determined as the
359 proportional coverage.

360 *Gyrodactylus gasterostei*. An isogenic line of *G. gasterostei*, derived from a single
361 individual collected at RBK in October 2014 was used for experimental infections.
362 Identification was based on morphology (Harris, 1982) and genomic sequencing
363 (region: GenBank AJ001841.1) (Harris *et al.*, 1999). Fish were individually
364 anaesthetized in 0.02% MS222. Then, using a dissecting microscope and fibre-optic
365 lighting, the caudal fins of an infected donor and recipient fish were overlaid until 2
366 individuals of *G. gasterostei* transferred to the caudal fin of the recipient. Infected fish
367 were screened 24 h p.i. in fresh water under anaesthesia (0.02% MS222) and body
368 surfaces checked for infection; fish uninfected after this initial examination were re-
369 infected. Subsequently, fish were screened every 5 days for 91 days in experiment 2
370 and every 4-5 days for 58 days in experiment 3. At the experimental endpoints fish
371 were killed, weighed and measured (as above), and dissected to record parasites in
372 the body cavity, swim bladder, gut, gills and eyes (the only co-infecting parasite

373 recovered was *S. solidus*). *G. gasterostei* is predominantly a parasite of external
374 body surfaces (>7000 fish examined from RBK have never contained this common
375 species in the branchial cavity; JC per. obs.).

376 *Thermal acclimation of parasites.* Source *Saprolegnia* and *Gyrodactylus* cultures
377 were maintained at a single intermediate temperature (15°C) prior to experiments to
378 provide infectious challenges with a standardized thermal reaction norm (given the
379 possibility of acclimation effects (Altman *et al.*, 2016)).

380 *Naturally-acquired infections persisting in experimental fish*

381 *Schistocephalus solidus* plerocercoid larva infections were refractory to the
382 prophylactic treatments described above and were the only naturally-acquired
383 macroparasite to carry over significantly into the experiments (*S. solidus* would have
384 been unable to transmit within experiments due to its indirect life cycle). Presence of
385 other macroparasites and overt microbial infections was confirmed to be at negligible
386 levels (<5% prevalence) through visual monitoring of experimental fish, direct
387 parasitological examination at endpoints (where sampling procedures allowed), and
388 by examination of animals prepared for, but unused in, experiments. The presence of
389 *S. solidus* infection was recorded in all experiments (see above) and included in
390 statistical analyses.

391 *Ethics*

392 Work involving animals conformed to U.K. Home Office (HO) regulations; elements
393 at Aberystwyth University were approved by the animal welfare committee of the
394 Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth
395 University and conducted in consultation with the HO inspectorate; elements at
396 Cardiff University were approved by the Cardiff University Ethics Committee and
397 conducted under HO licence PPL 302357.

398 *Gene expression measurements*

399 We measured expression of 14 immune-associated genes (Table 1) using
400 quantitative real-time PCR as previously described (Hablützel *et al.*, 2016). The
401 immunological roles of the genes are summarized in Table S1.

402

403 *Analyses*

404 All analyses were carried out in *R* version 3.2.3. In the statistical analysis of our
405 experimental results we employed linear mixed models (LMMs, package *lme4*) or
406 general linear models (LMs) for the confounder-adjusted analysis of gene expression
407 responses (the latter if no random term was significant). Power transformations
408 derived via a Box-Cox procedure were applied to individual expression variables on
409 a case-by-case basis following evaluation of standard model diagnostics. In a few
410 cases skewed gene expression variables containing some zeros were analysed in
411 generalised linear models for location, scale and shape (Rigby & Stasinopoulos,
412 2005, Stasinopoulos & Rigby, 2007) (GAMLSS) with a zero-adjusted gamma
413 distribution (using the package *gamlss*). For *Saprolegnia* infections we considered
414 the proportion of body surface coverage by erupted mycelia and analysed these data
415 in GAMLSS models. The latter employed a zero-inflated beta distribution
416 incorporating parameters for the probability (α) of not developing overt symptoms
417 (erupted mycelia) and also for the severity of symptoms (location parameter, μ ,
418 reflecting coverage by mycelia in overt cases). For *Gyrodactylus* we considered
419 demographic parameters for continuously monitored individual infrapopulations (time
420 to peak infection and peak infection abundance) analysing these data in LMs with a
421 $(\log_{10} + 1)$ transformation. *Schistocephalus* infection data (total infection weight per
422 host, parasitic index [total infection weight / host weight]) were analysed in LMs, or in
423 generalized additive models (GAM) (Wood, 2006) when irregular trends were better
424 represented by non-parametric smoothers (package *mgcv*) (random intercept terms
425 for tank were not significant in these analyses). Except where otherwise stated,
426 statistical analyses of gene expression and infection metrics included explanatory
427 terms for the following in starting models: host length, sex, body condition (calculated
428 as residuals from a quadratic regression of weight on length), *Schistocephalus*
429 infection if this was present in the sample (present/absent; and except where this
430 infection was the analysed response), reproductive condition (breeding / non-
431 breeding condition; only in the long-term experiment 3), factorial experimental
432 treatments and experimental block (experiment 1) or year (experiment 3); sampling
433 (tank) and assaying (assay plate) structure was represented with random intercept
434 terms, where relevant. Interaction terms of interest were included where specified
435 below. The model for *Saprolegnia* infection in experiment 2 was developed using just

436 the thermal treatment terms and host terms significant in experiment 1, due to limited
 437 sample size. Models for gene expression in experiments 1 and 2 included factors
 438 representing exposure and overt infection with *Saprolegnia*; the experiment 2
 439 analysis contained a fixed term for time (in degree days) within the experiment.
 440 Random terms were assessed (in the full model) by likelihood ratio tests in LMMs
 441 and GAMLSSs. When a random effect was added to a GAM as penalized regression
 442 terms (to give a generalised additive mixed model, GAMM), its importance was
 443 assessed by Akaike information criterion (AIC). Fixed model terms were retained
 444 based on AIC for LMs, GAMLSSs and GAMs and *F*-tests (with Satterthwaite's
 445 approximation to degrees of freedom) for LMMs. Reported *P* values were
 446 determined by likelihood ratio tests in GAMLSSs, *F* tests in LMs, *F* tests with
 447 Satterthwaite's approximation in LMMs and Wald tests in GAMs. Standard diagnostic
 448 plots of residual and fitted values and quantile-quantile plots of residuals were
 449 inspected for all models.

450 A sinusoid model (1) was employed to explicitly represent the possibility that the
 451 direct thermal effect on resistance to *Saprolegnia* (α ; probability of resisting overt
 452 infection following exposure), as observed in laboratory experiments 1 and 2, was
 453 counteracted by other seasonal environmental influences on host
 454 immunocompetence in experiment 3:

$$455 \quad \textit{Saprolegnia} \alpha = x + \text{Immunocompetence driver (ID)} + \text{Thermal driver (TD)} \quad (1)$$

$$456 \quad \text{ID} = c \times a \times \cos \left[\left(\frac{2\pi t}{12} \right) - \theta^1 \right]$$

$$457 \quad \text{TD} = d \times E$$

$$458 \quad E = b \times \cos \left[\left(\frac{2\pi t}{12} \right) - \theta^2 \right]$$

459 Where *E* is environmental temperature (°C), *Saprolegnia* α is the monthly probability
 460 of resisting overt *Saprolegnia* symptoms and *t* is time (months) (all observed in
 461 experiment 3); parameters are detailed in Table 1. Given the seasonal nature of
 462 temperature and *Saprolegnia* α variation in experiment 3, this model represents a
 463 temperature driver (TD) and a putative immunocompetence driver (ID) with separate
 464 (superimposed) annual sinusoid functions (Stolwijk *et al.*, 1999). We parameterized

465 the amplitude and acrophase of TD from our records of temperature (using
466 parameter estimates from cosinor regression of temperature against time, see
467 below) and the thermal coefficient, d (converting temperature into α , see Table 1),
468 from laboratory experiments (using an intermediate value based on analysis of
469 experiments 1 and 2). Taking an inverse modelling approach we then fitted this
470 partially parameterized model (1) to the monthly *Saprolegnia* α data (from
471 experiment 3) and estimated parameters associated with ID. For this we used
472 package *FME* (Soetaert & Petzoldt, 2010) to carry out constrained fitting of the
473 model. Cosinor regression (Tong, 1976) was carried out with package *cosinor* in
474 order to estimate the amplitude and acrophase of seasonal temperature variation.

475

476

Parameter	Definition	Estimate	Method of estimation
x	Constant	1.28 ± 0.37	Constrained fitting of <i>Saprolegnia</i> α data to (1)
c	Immunocompetence coefficient		
a	Amplitude of immunocompetence driver		
k	$c \times a$	2.74 ± 0.53	Constrained fitting of <i>Saprolegnia</i> α data to (1)
Θ^1	Acrophase of immunocompetence driver	1.28 ± 0.29	Constrained fitting of <i>Saprolegnia</i> α data to (1)
d	Thermal coefficient	-0.375	Intermediate value from GAMLSS models (experiments 1 and 2)
b	Amplitude of thermal driver	5.02 ± 0.27	Cosinor regression of environmental temperature (E) on time (t)
Θ^2	Acrophase of thermal driver	1.30 ± 0.05	Cosinor regression of E on t

477

478

479 **Table 1** Parameters from sinusoid model of *Saprolegnia* α variation in experiment 3.

480

481 As descriptors of thermal variability in the 7-day windows preceding sampling points
482 in experiment 3 we considered temperature variance, amplitude of diel temperature
483 variation, the shape of the time series represented by Fourier coefficients, and the
484 maximum upward trend (given that in experiment 1 we observed a protective effect
485 of upward temperature shifts). To quantify diel temperature variation we fitted a GAM
486 to each time series, with parametric sinusoidal time terms to represent diel oscillation
487 and a non-parametric smoother for time to represent other temporal trends (Wood,
488 2006). Amplitude of the diel oscillation was calculated from the parameters of the
489 sinusoidal terms (Stolwijk *et al.*, 1999). Between-month distances based on Fourier
490 coefficients (FCD) were calculated from centred time series using package *TSdist*
491 (Mori *et al.*, 2017).

492

493 **Results**

494 *The prevailing temperature consistently had substantial effects on infection and*
 495 *immunity under controlled laboratory conditions*

496 Both experiments 1 and 2 included factorial combinations of prevailing and lagged
 497 thermal treatments. Considering the main effects of prevailing temperature first, we
 498 found that most immune-associated genes (12/14) (Fig. 3a, Table 2; Fig. S2) showed
 499 significant change in expression across the range 7-23°C (experiment 1) and many
 500 (6/14) (Fig. 3a, Table 3; Fig. S3) did across the range 7-15°C (experiment 2). These
 501 expression changes were consistent with monotonic responses (Fig. S2-S3). The
 502 broad effect size of prevailing temperature on gene expression was substantial:
 503 temperature variation across the range 7-23°C had a similar impact to sex and
 504 greater impact than other host variables such as size, body condition and infection
 505 status (Fig. S4).
 506

Gene	Model type	T (7-23°C)		ΔT (-8, 0, +8°C shift)	
		Parameter	<i>P</i>	Parameter	<i>P</i>
<i>cd8a</i>	LM	0.009±0.001	1.7×10^{-8}		
<i>ighm</i>	LM	0.007±0.001	5.5×10^{-11}	-0.004±0.001	4.0×10^{-5}
<i>ighz</i>	GAMLSS	α -0.096±0.045	0.028	α 0.012±0.050	0.009
<i>foxp3b</i>	LM	0.009±0.002	9.6×10^{-6}	-0.006±0.002	0.009
<i>il4</i>	LMM	0.0004±0.0002	0.037		
<i>il17</i>	LMM	-0.002±0.001	0.035		
<i>orai1</i>	LMM	-0.003±0.001	2.5×10^{-5}		
<i>tirap</i>	LM	0.009±0.001	5.7×10^{-13}		
<i>tbk1</i>	LMM	-0.0014±0.0002	2.8×10^{-12}		
<i>il1r1</i>	LMM	0.005±0.002	0.004	-0.005±0.002	0.010
<i>lyz</i>	LM	0.010±0.002	2.1×10^{-6}		
<i>defbl2</i>	LM	0.008±0.002	2.4×10^{-4}		

507

508 **Table 2** Significant effects of thermal regimen on immune gene expression in
 509 experiment 1. Parameters and *P* values for prevailing temperature (T) and prior
 510 thermal shift (ΔT). T and ΔT are represented as continuous variables; no additional
 511 genes were found to be thermally-dependent through representing T and ΔT with
 512 quadratic terms. Data were analyzed in confounder-adjusted general linear models
 513 (LM), linear mixed models (LMM) and generalized additive models for location, scale

514 and shape (GAMLSS). Genes without significant effects for T or ΔT are omitted;
 515 there were no significant T \times ΔT effects. Note that for the GAMLSS model above the
 516 parameter sign is opposite to the direction of the biological effect.

517

Gene	Model type	T (7-15°C)		WL (0-3 months at 7°C)	
		Parameter	<i>P</i>	Parameter	term <i>P</i>
<i>ighm</i>	LMM			1 mo -0.012±0.006	0.013
				2 mo -0.019±0.006	
				3 mo -0.016±0.007	
<i>il17</i>	LM	-0.005±0.003	0.090	3 mo 0.077±0.027	0.020
<i>il12ba</i>	LM	0.019±0.007	0.005		
<i>orai1</i>	LM	-0.015±0.004	0.001		
<i>tbk1</i>	LMM	-0.010±0.002	9.1 × 10 ⁻⁶		
<i>il1r1</i>	LMM			2 mo 0.014±0.007	0.002
				3 mo 0.028±0.008	
<i>defbl2</i>	LM	0.016±0.005	0.002		
<i>gpx4a</i>	LMM			1 mo 0.0035±0.0018	0.011
				2 mo 0.0061±0.0019	
				3 mo 0.0056±0.0020	

518

519

520 **Table 3** Significant effects of thermal regimen on immune gene expression in
 521 experiment 2. Parameters and *P* values for prevailing temperature (T) and simulated
 522 prior winter length (WL). T is represented as a continuous variable (no additional
 523 genes were found to be dependent on T through adding a quadratic term); WL is
 524 represented as a factor as differences were associated with any simulated winter
 525 exposure or only with longer exposures. Data were analyzed in confounder-adjusted
 526 general linear models (LM), linear mixed models (LMM) and generalized additive
 527 models for location, scale and shape (GAMLSS). Genes without significant effects
 528 for T or WL are omitted; there were no significant T \times WL effects.

529

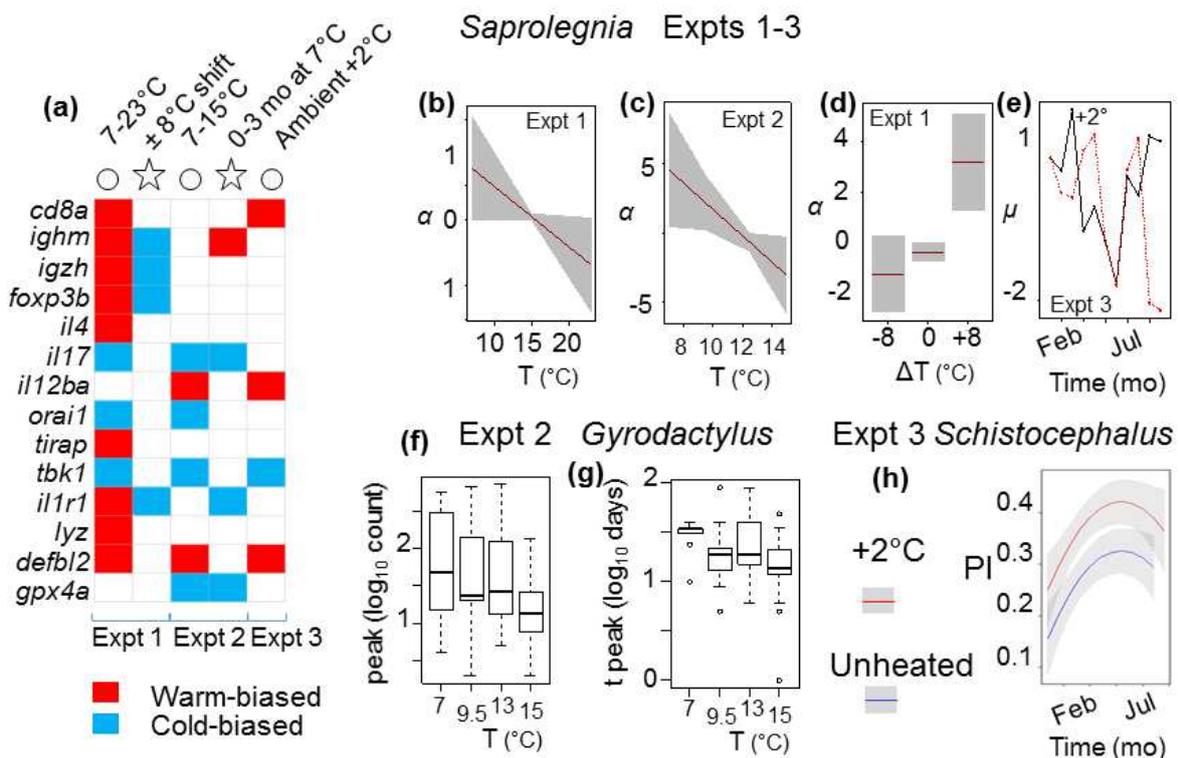
530 In *Saprolegnia* challenges (Fig. 3b-c), resistance to overt disease (α parameter)
 531 became less probable with increasing prevailing temperature in both laboratory
 532 experiments (GAMLSS analyses; experiment 1, α = -0.12±0.04, *P* = 2.9 × 10⁻³,
 533 experiment 2, α = -1.05±0.46, *P* = 1.1 × 10⁻⁵). In *Gyrodactylus* challenges in
 534 experiment 2, low temperature exposure during the early stages of established

535 infection produced a more severe outcome: parasite abundance peaking later and
 536 higher (Fig. 3f, g) (LMs; \log_{10} time to peak = -0.04 ± 0.01 , $P = 6.1 \times 10^{-3}$; \log_{10} peak
 537 population = -0.07 ± 0.02 , $P = 9.5 \times 10^{-4}$). Notably, data presented by Harris (1982)
 538 indicate that *G. gasterostei* infrapopulations also peak later and higher when
 539 maintained at a constant temperature of 10 compared to 15°C. The direction of these
 540 thermal effects on peak parasite numbers is contrary to the expectation that such a
 541 temperature increase would promote *Gyrodactylus* population growth in permissive
 542 conditions (Harris, 1982; Gelnar, 1990; Jackson & Tinsley, 1994; Sereno-Uribe *et*
 543 *al.*, 2012), and indicative that low temperature impairs the early development of
 544 resistance responses (Andersen & Buchmann, 1998).

545

546

547



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549

550

551 **Fig. 3** Effects of prevailing temperature and past temperature change on gene
 552 expression and disease progression in experiments. (a) Colour matrix showing

553 significant gene expression responses to temperature regimens in experiments 1, 2
554 and 3 (see key). Open circles indicate responses to prevailing temperature and stars
555 responses to previously experienced temperature change (i.e., lagged effects). As
556 expected, the numbers of genes responding detectably to prevailing temperature fell
557 with the thermal range examined in the respective experiments (experiment 1, 16°C
558 range: 12/14 responsive genes; experiment 2, 8°C range: 6/14 responsive genes;
559 experiment 3, 2°C range: 4/14 responsive genes). There was consistency across
560 experiments in the sign of significant responses to prevailing temperature, which
561 were always the same for a given gene (10 comparisons). Fewer genes (< half the
562 number) responded detectably to lagged temperature effects than to prevailing
563 temperature across experiments 1 and 2. For lagged effects shown in (a), genes are
564 termed cold-biased if they had higher expression than expected following a
565 downwards temperature shift (experiment 1) or if they responded positively to
566 increasing winter length (experiment 2). (b-e) Significant responses of *Saprolegnia*
567 infection outcome to thermal regimen in experiments 1-3; plots (on the scale of the
568 model linear predictor) show confounder-adjusted effects from generalized additive
569 models for location, scale and shape (GAMLSS) with 95% confidence intervals
570 (shaded). In experiments 1 (b) and 2 (c) the probability of not developing overt
571 symptoms (α) decreased with increasing prevailing temperature. There was a
572 protective residual effect of a recent +8°C temperature shift in experiment 1 (d). In
573 experiment 3 symptom severity (μ) was subject to a time \times temperature treatment
574 (+2°C) interaction (e). (f-g) Significant responses of *Gyrodactylus* infrapopulation
575 dynamics in experiment 2. Lower initial exposure temperature (shown on the x –
576 axis) resulted in infections with higher (f) and later (g) abundance peaks (*peak*,
577 highest count; *t peak*, time to reach highest count). Box-and-whisker plots show log-
578 transformed data for individual infrapopulations (only exposure temperature was
579 significant in statistical models). (h) Response of *Schistocephalus* parasitic index
580 (infection weight / host weight, PI) to a +2°C manipulation across the year in
581 experiment 3 (outside mesocosms). PI was significantly greater in hosts from heated
582 mesocosms. Lines are confounder-adjusted effects from a general linear model (LM)
583 with 95% intervals (shaded).

584

585 *Lagged effects of past temperature on infection and immunity were detectable but*
586 *not consistently important*

587 Some main effects of lagged thermal treatments were evident in the gene expression
 588 results in both laboratory experiments (Fig. 3a, Tables 2-3). However, lagged thermal
 589 effects occurred much less frequently (Fig. 3a) than prevailing temperature effects
 590 (14 genes showed significant prevailing effects and 6 genes significant lagged
 591 effects in one or both of experiment 1 and 2). There were no effects on gene
 592 expression due to interactions between prevailing temperature and preceding
 593 temperature treatments in either experiment.

594

595 There were no lagged main effects of temperature on *Saprolegnia* infections in
 596 experiments 1 or 2. This was with the exception of a single scenario: where rapid
 597 upward shifts in temperature in experiment 1 had a protective effect (increasing α)
 598 (Fig. 3d) (GAMLSS analysis; +8°C shift $\alpha = 3.92 \pm 1.20$, reference level = -8°C shift;
 599 term deletion $P = 1.1 \times 10^{-4}$). For *Gyrodactylus* in experiment 2 we found no effect of
 600 past temperatures previous to the period of infection (i.e., of simulated winter length)
 601 on infrapopulation dynamics. No interactions occurred between lagged temperature
 602 and prevailing temperature treatments for *Saprolegnia* (experiments 1-2) or
 603 *Gyrodactylus* (experiment 2).

604 *Thermal effects on infection and immunity were readily detectable in a realistic*
 605 *warming scenario superimposed upon natural environmental cycles*

606 Turning to our mesocosm experiment we first asked what effect the +2°C
 607 manipulation (simulating climate warming) had on gene expression and infection
 608 outcomes. We found that several genes responded significantly (*cd8a*, *il12ba*,
 609 *defbl2*, *tbk1*; always in the same direction as responses in laboratory experiments),
 610 even against the background of natural seasonal variation (Fig. 3a; Table 4). For
 611 *Schistocephalus* infections *in situ* within the mesocosms, the direct effect of the +2°C
 612 increment increased the parasitic index (infection weight/host weight, PI) (Fig. 3h)
 613 (LM; +2°C 0.095 ± 0.023 , $P = 1.1 \times 10^{-4}$) and plerocercoid weight (GAM; +2°C
 614 10.9 ± 4.6 , $P = 0.02$) although without the extreme plerocercoid size increases
 615 reported in recent constant temperature experiments (Macnab & Barber, 2012).
 616 There was no lagged main effect of the +2°C temperature manipulation on
 617 *Saprolegnia* and *Gyrodactylus* infection outcomes in fish extracted from the
 618 mesocosms and equalized to the same (natural) temperature regimen before
 619 exposure to infection. However, there was a significant month \times lagged temperature

620 treatment interaction for symptom severity in *Saprolegnia* (μ parameter), with
 621 modulated infection outcomes in the winter and late summer (Fig. 3e) (GAMLSS;
 622 $+2^{\circ}\text{C} \times \text{month}$: Feb^{low} -1.69 ± 0.81 , Aug^{low} 2.400 ± 1.16 , Sept^{low} -3.90 ± 1.15 ; term
 623 deletion $P = 7.9 \times 10^{-4}$).

624

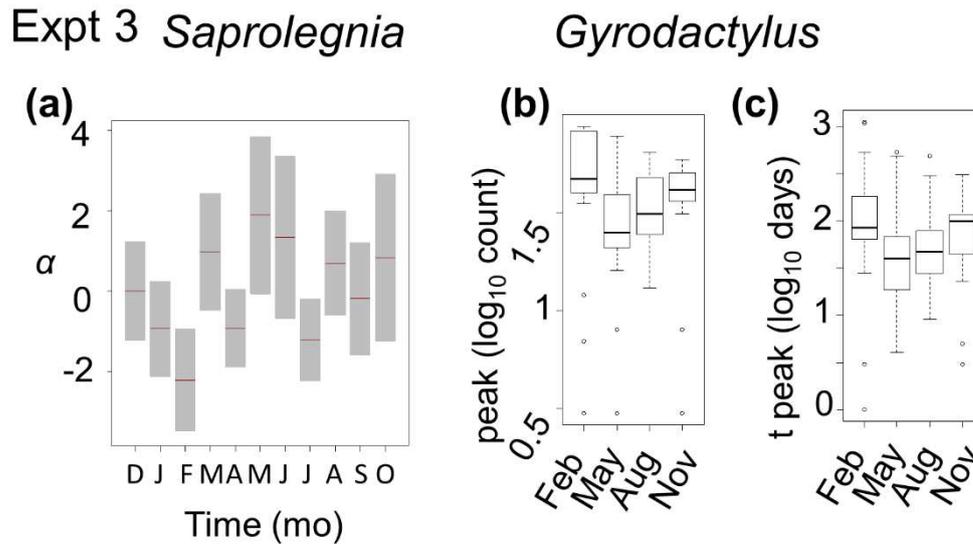
Gene	Model type	Parameter ($+2^{\circ}\text{C}$)	P
<i>cd8a</i>	LMM	0.0248 ± 0.0122	0.042
<i>il12ba</i>	LM	0.0804 ± 0.0209	1.7×10^{-4}
<i>tbk1</i>	LMM	-0.0713 ± 0.0194	2.7×10^{-4}
<i>defbl2</i>	LMM	0.0012 ± 0.0003	4.2×10^{-5}

625

626 **Table 4** Significant effects of thermal regimen on immune gene expression in
 627 experiment 3. Parameters and P values for thermal treatment (unheated / $+2^{\circ}\text{C}$).
 628 Data were analyzed in confounder-adjusted general linear models (LM), linear mixed
 629 models (LMM) and generalized additive models for location, scale and shape
 630 (GAMLSS). Genes without significant effects for thermal treatment are omitted.

631 *Given thermal responses observed in the laboratory, disease progression was*
 632 *paradoxically highest in winter in an environment with natural seasonality*

633 We next asked how well the year-round patterns of infection susceptibility seen in
 634 mesocosms (experiment 3) corresponded to the observed responses in our
 635 laboratory manipulations of temperature. In the more realistic mesocosm setting
 636 there was striking evidence that seasonal trends were superimposed upon direct
 637 thermal effects, leading to results unpredictable on the basis of thermal variation
 638 alone (Zimmerman *et al.*, 2010). Thus, the probability of resisting overt *Saprolegnia*
 639 infection (α parameter), which decreased when temperature was increased in the
 640 laboratory (Fig. 3b, c), paradoxically was lowest during winter in the mesocosms
 641 (Fig. 4a) (GAMLSS; α Feb -2.49 ± 0.79 ; month term deletion, $P = 1.8 \times 10^{-4}$). A
 642 corresponding pattern was seen in *in situ* *Schistocephalus* infections in the
 643 mesocosms. As described above (see also Fig. 3h), the $+2^{\circ}\text{C}$ temperature



644

645 **Fig. 4** Greater disease progression (following challenge infections) in winter in an
 646 outdoors seasonal environment (experiment 3). (a) For *Saprolegnia*, probability of
 647 not developing overt symptoms (α) was significantly variable in time and lowest in
 648 February; plot shows confounder-adjusted effects from a generalized additive model
 649 for location, scale and shape (GAMLSS) with 95% confidence intervals shaded (on
 650 the scale of the model predictor). (b-c) *Gyrodactylus* infrapopulations monitored
 651 through winter months (starting in November or February, compared to May or
 652 August) had higher (b) and later (c) abundance peaks (*peak*, highest count; *t peak*,
 653 time to reach highest count). Box-and-whisker plots show log-transformed data for
 654 individual infrapopulations (only exposure month was significant in statistical
 655 models).

656

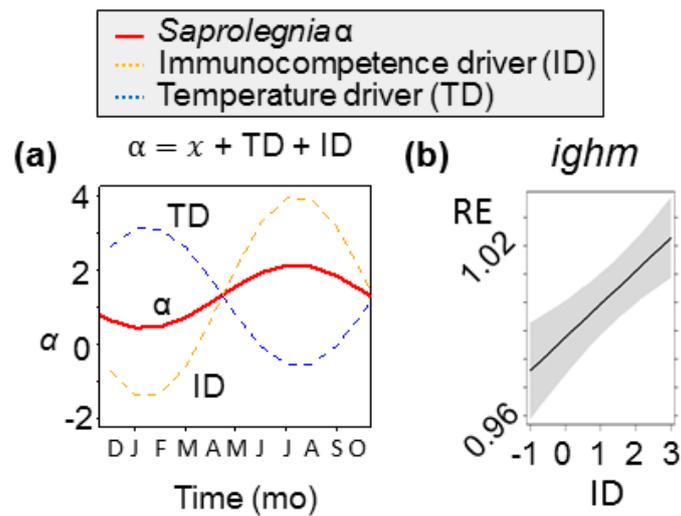
657 manipulation produced an increase in PI, indicating a positive thermal dependence
 658 of disease severity (as for *Saprolegnia* α). Contrary to this thermophilic trend,
 659 though, PI in fact increased during the winter months (Fig. 3h) and ceased to
 660 increase thereafter (LM with quadratic term for time; time 0.056 ± 0.015 , $P = 0.014$;
 661 time² -0.004 ± 0.001 , $P = 2.7 \times 10^{-3}$). This pattern is consistent with lowered host
 662 resistance during winter and rapid plerocercoid growth (relative to the host) despite
 663 low winter temperatures. For both *Saprolegnia* and *Schistocephalus*, the pattern of
 664 results is thus suggestive of a seasonal immunocompetence variable (low host
 665 immunocompetence in winter) that acts in opposition to the direct effects of

666 prevailing environmental temperature (positive thermal dependence of host
 667 exploitation, as demonstrated in experiments 1 and 2). For *Gyrodactylus*, as for
 668 *Saprolegnia* and *Schistocephalus*, the worst disease also occurred in winter (Fig. 4b,
 669 c), with infection abundance peaking later (LM; \log_{10} time to peak, Aug 0.14 ± 0.09 ,
 670 Nov 0.21 ± 0.08 , Feb 0.30 ± 0.077 , reference May; month term deletion $P = 9 \times 10^{-4}$)
 671 and higher (LM; \log_{10} peak population; Aug 0.17 ± 0.15 , Nov 0.32 ± 0.14 , Feb
 672 0.43 ± 0.12 ; $P = 0.007$).

673 *A latent seasonal immunocompetence variable, that correlated with immune gene*
 674 *expression and opposed thermal effects, explained winter-biased disease*
 675 *progression in natural circumstances*

676 We set out to explicitly partition seasonal thermal and immunocompetence effects
 677 contributing to the winter-biased pattern of infection susceptibility seen in experiment
 678 3. We focussed on *Saprolegnia*, for which most experimental data were available
 679 and for which the binary infection endpoint (α) simplified interpretation. As seasonal
 680 fluctuation can be represented with sinusoid functions (Stolwijk *et al.*, 1999), we
 681 constructed a model explaining the (logit scale) *Saprolegnia* α parameter in terms of
 682 a cosine wave for annual thermal variation and another cosine wave for seasonally-
 683 varying immunocompetence (see (1), Table 1, Fig. 5). We first parameterized the
 684 amplitude and acrophase of the annual temperature function from our 2014-2015
 685 temperature monitoring data and estimated the coefficient converting this into
 686 infection rate from observations on the effect of prevailing temperature in
 687 experiments 1 and 2. (We did not include lagged thermal effects because of the lack
 688 of these in experiments 1 and 2, except for the protective effect of previous sharp
 689 warming; although we do examine the latter, and other aspects of thermal variance,
 690 further below.) We then used an inverse modelling approach to compute the
 691 parameters of the latent immunocompetence function by fitting the partially
 692 parameterized model to our 2014-2015 *Saprolegnia* infection data. The fully
 693 parameterized model explained 22% of the variation in *Saprolegnia* α , and
 694 suggested that effects driven by temperature and by seasonal immunocompetence
 695 were almost collinear (Fig. 5). Importantly, we note that the distinct contributions of
 696 temperature and immunocompetence would therefore have been unobservable had
 697 only infection data been available (as in many field studies).

698



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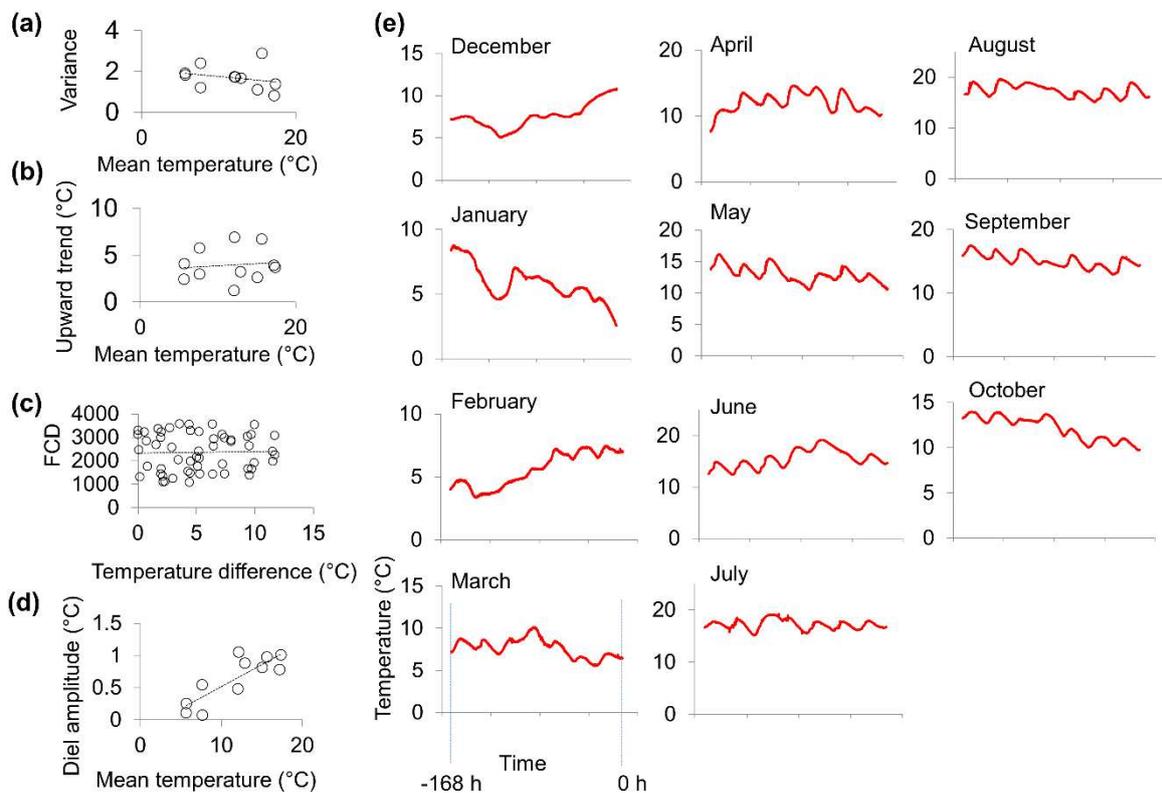
700 **Fig. 5** A latent immunocompetence variable, which independently correlates with
 701 seasonal expression in immunity genes, reconciles observations from laboratory and
 702 outdoors mesocosm experiments. (a) Results of an inverse model of observed
 703 variation in *Saprolegnia* α in experiment 3: α is explained via the superimposition of a
 704 sinusoidal seasonal temperature driver, TD (parameterized from observed
 705 relationships with temperature in the laboratory and from field temperature records),
 706 and a hypothetical (latent) sinusoidal immunocompetence variable, ID
 707 (parameterized by constrained fitting of α data to the model); x is a constant. (b) The
 708 association of the latent immunocompetence variable from the analysis shown in (a)
 709 with *ighm* relative expression (RE) in experiment 3; line shows confounder-adjusted
 710 effect (on the scale of the model linear predictor) from a linear mixed model (LMM)
 711 with random intercepts for month; 95% confidence interval shaded.

712

713 We considered whether the latent immunocompetence variable derived above might
 714 represent the protective lagged effect of sharp temperature rises, as observed in
 715 experiment 1, or of other aspects of preceding temperature variability, but found this
 716 to be unlikely. As immunocompetence and prevailing temperature were collinear
 717 (see above), we expected that any component of temperature variability
 718 predominantly driving immunocompetence would necessarily be correlated with

719 prevailing temperature. Therefore, we examined different descriptors of temperature
 720 variability (in the week before monthly sample points) for this correlation.

721



722

723 **Fig. 6** Association between descriptors of temperature variability and mean
 724 temperature in outdoors mesocosms. (a) variance vs mean temperature; (b)
 725 maximum upward trend vs mean temperature; (c) pairwise month-to-month
 726 distances between time series shapes (Fourier coefficient distances, FCDs) vs
 727 pairwise month-to-month temperature differences; (d) amplitude of diel temperature
 728 variation vs mean temperature. Analyses shown above are based on the final 7-day
 729 period fish spent in the mesocosm habitats prior to monthly exposures to
 730 *Saprolegnia* in the 2014-2015 run of experiment 3. Panel (e) shows monthly
 731 temperature trajectories for the 7-day period analyzed, from -168 to 0 h.

732

733 The maximum upward trend, variance and shape (FCD) of monthly temperature time
 734 series (in the week before sampling) were not associated with mean monthly

735 prevailing temperature (Fig. 6a-c). Although the amplitude of diel temperature
736 variation did increase with temperature (Fig. 6d), the absolute size of this increase
737 was small ($\sim 1^\circ\text{C}$ across the annual thermal range; corresponding to a $\sim 2^\circ\text{C}$ diel
738 range difference) when considered in the light of the effect size for a $+8^\circ\text{C}$ shift on
739 *Saprolegnia* α in experiment 1. The latter corresponded to a change in α of 0.8
740 across 2°C (the annual diel range difference), compared to an annual α range of >5
741 for the immunocompetence driver shown in Fig. 5a.

742 We also asked whether the latent immunocompetence variable was associated with
743 independent data for the expression of immunity genes. We found that one gene,
744 *ighm* ($P = 0.003$) (Fig. 5b), was clearly associated and that four others were more
745 marginally associated: *il4* ($P = 0.07$), *tirap* ($P = 0.04$), *defbl2* ($P = 0.06$) and *cd8a* (P
746 $= 0.06$) (in confounder-adjusted LMMs, with random intercepts for month). In all of
747 these cases, increased expression corresponded to increased latent
748 immunocompetence. The association with *ighm* is consistent with the suspected
749 involvement of antibodies in resistance to *Saprolegnia* infection (Minor *et al.*, 2014)
750 and with elevated early autumn anti-*Saprolegnia* antibody seropositivity in wild
751 salmonids (Fregeneda-Grandes *et al.*, 2009).

752

753 Discussion

754 We focussed on the three-spined stickleback and its pathogens as a natural
755 experimental model. We readily detected perturbation of immune expression and
756 infectious disease progression in a realistic experimental climate warming scenario
757 applied in naturalistic outdoors mesocosms. Even for a modest thermal increment
758 ($+2^\circ\text{C}$), significant expression differences were observed for 4/14 immune-
759 associated genes examined (*cd8a*, *tbk1*, *il12ba*, *defbl2*) whilst *Schistocephalus*
760 parasitic index and plerocercoid growth increased. Lagged thermal effects on
761 *Saprolegnia* symptom severity (μ) also featured in a significant interaction with
762 month. This interaction reflected a distinctive seasonal pattern of disease
763 progression in the warmed environment, demonstrating the potential for change in
764 the phenology of disease (Buehler *et al.*, 2008; Paull & Johnson, 2014) under
765 climate warming.

766 In CBVs like the three-spined stickleback, within-host infection dynamics can thus be
767 expected to respond appreciably to rapid year-on-year warming. Direct thermal
768 effects may drive part of this response, which in turn contributes to
769 population- (Barber *et al.*, 2016; Mignatti *et al.*, 2016) and community-
770 level (Karvonen *et al.*, 2013; Paull & Johnson, 2014) pathogen dynamics. But these
771 higher-level responses will also depend on other factors: on thermal responses of
772 free-living transmission stages and on indirect effects of temperature (on both within-
773 host and free-living stages) mediated through changes in the environment. It is
774 important (as we describe below in the case of thermal and non-thermal
775 environmental influences on *Saprolegnia* disease progression) to decompose such
776 complex composite processes into their fundamental parts, if we are to understand
777 the sources of dynamical change in natural systems.

778 To estimate thermal effects (holding other environmental effects constant) we carried
779 out laboratory experiments with factorial combinations of lagged and prevailing
780 temperature treatments. The controlled conditions in these experiments would have
781 prevented the formation of seasonal environmental variation (e.g., plankton
782 development) as occurred in the mesocosm experiment. The laboratory
783 experiments, together with the mesocosm experiment (above), not unexpectedly (Bly
784 & Clem, 1992; Maniero & Carey, 1997; Le Morvan *et al.*, 1998; Makrinos & Bowden,
785 2016) confirmed a major general effect of temperature in modulating immunity and
786 within-host infectious disease outcomes in CBVs. All of the 14 gene expression
787 measures and all 3 infection systems that we examined showed some significant
788 response to experimental manipulation of temperature, in many cases with
789 substantial effect sizes. Whilst other studies of ectothermic organisms have
790 emphasized the importance of lagged thermal influences on immunity, we found that
791 thermal effects were mediated most powerfully by the prevailing temperature.
792 Overall, less than half the number of genes (in experiments 1 and 2) showed
793 expression responses to past thermal variation as to prevailing temperature. All three
794 of our infection systems showed the effect of temperature prevailing within the
795 timeframe of infection, but there were few cases in which temperature prior to this
796 timeframe was important. Amongst the lagged thermal treatments in our laboratory
797 experiments only sharp temperature rises had any significant effect: decreasing the
798 probability of developing of overt *Saprolegnia* infection. As discussed above, there

799 was also an interaction between lagged thermal treatment and *Saprolegnia* symptom
800 severity (μ) in the mesocosm experiment. Putting these results in perspective, we
801 note that the lagged temperature treatments we used in laboratory experiments
802 (simulated winters 0-3 months long and 8°C thermal shifts over 6 h) were relatively
803 extreme. This would have exaggerated the importance of lagged compared to
804 prevailing temperature effects, as the latter were represented in our experiments by
805 a set of values well within the natural range. Interestingly we did not find an anti-
806 protective effect of sharp temperature falls on *Saprolegnia* infection. Whilst such a
807 tendency has been reported in saprolegniosis of channel catfish (Bly *et al.*, 1992),
808 and in fungal infections of lower vertebrates (Raffel *et al.*, 2013), our results suggest
809 this effect is not a general one. Even leaving the effects of the non-thermal variation
810 (see below) aside, our data indicate that past temperature records will be of limited
811 use for managers of CBV populations in projecting infectious disease susceptibility.
812 Rather systems for the projection of disease risk based on prevailing temperature
813 variation will be more effective.

814

815 Combining our mesocosm and laboratory experimental data we considered the
816 contributions of thermal and non-thermal environmental variation to disease
817 progression. Importantly, in the outdoors mesocosm environment (subject to biotic
818 and abiotic seasonality), *Saprolegnia* and *Schistocephalus* infections occurred in a
819 pattern not explained by their responses to experimental thermal manipulations. In
820 both infections disease progression was increased by upwards experimental
821 manipulation of temperature, all other things being equal, but under mesocosm
822 conditions was also at its greatest in winter. Crucially, our study design allowed us to
823 partition thermal effects from other environmental effects on disease progression,
824 revealing their relative magnitude. Using an inverse modelling approach to represent
825 monthly *Saprolegnia* challenge infection outcomes in the outdoor mesocosms, and
826 including (prevailing) thermal effects parameterized from our laboratory experiments,
827 we were able to derive a seasonal latent variable opposing (and slightly
828 outbalancing) thermal effects. This variable represented environmental effects on
829 anti-*Saprolegnia* immunocompetence, other than those due to the prevailing
830 temperature, and reconciled laboratory and mesocosm observations. It could not be
831 explained by seasonal patterns of temperature variance (cross-referencing to effects
832 observed in laboratory experiments), and was independently (positively) correlated

833 with monthly expression of the immunoglobulin M heavy chain gene *ighm*. This is of
834 note because of the likely relevance of IgM for resistance to *Saprolegnia* (Minor *et*
835 *al.*, 2014). Furthermore, as teleost IgM antibodies may have a short half-life (1-3
836 days) (Voss Jr *et al.*, 1980; Ye *et al.*, 2010, 2013), a link between levels of heavy
837 chain mRNA and functional antibody is not unrealistic.

838 Thus, the non-thermal environmental contribution (via seasonal immunocompetence
839 effects) to *Saprolegnia* disease progression variance is large (of similar size to the
840 thermal contribution, slightly outbalancing it across the year). Whilst it is beyond the
841 scope of the present study to determine the environmental agents involved, such
842 seasonal variation in immunity is well known in other vertebrate systems
843 (Beldomenico *et al.*, 2008; Martin *et al.*, 2008). It should be pointed out, moreover,
844 that although some seasonal variation in the expression of immunity genes occurs in
845 mesocosm fish, we have previously observed such responses to be diminished
846 compared to those in the wild (Hablützel *et al.*, 2016). This suggests that the
847 component of disease progression variation determined by non-thermal
848 environmental effects on immunocompetence, and not directly by temperature, may
849 be even larger under fully natural conditions in the wild.

850

851 We note, additionally, the variable sign in the disease responses of our 3 infection
852 systems to prevailing temperature manipulations (positive for *Saprolegnia* α and
853 *Schistocephalus* parasitic index and negative for *Gyrodactylus* abundance). This is
854 consistent with the simple theoretical scenario, introduced at the beginning, where
855 disease worsens or ameliorates determined by the interplay of species-specific
856 thermal reaction norms in host and pathogen (Jackson & Tinsley, 2002). Whilst some
857 previous studies have emphasized the magnifying effects of warming temperature
858 regimens on host susceptibility in specific systems (Macnab & Barber, 2012), it is
859 also possible to find examples where rising temperature increases
860 resistance (Jackson & Tinsley, 2002; Douglas *et al.*, 2003; Raffel *et al.*, 2013).
861 Furthermore, in other cases infectious disease may show convex responses to
862 temperature, for example with symptoms emerging across a limited temperature
863 range (Gilad *et al.*, 2003; Ito & Maeno, 2014). This can result from non-linear
864 thermal reaction norms in host and or parasite. Thus, although thermal change, all
865 other things being equal, readily shifts the burden of disease caused by individual

866 pathogen species, the direction of these shifts may not be consistent, and the overall
867 disease outcome in host-parasite communities is likely to play out in a system
868 specific way.

869

870 Elements of our results also provide an additional perspective to those of (Dittmar *et*
871 *al.*, 2014) who examined head kidney (HK) cell responses and immune gene
872 expression in *G. aculeatus* under different thermal regimens and with an emphasis
873 on the upper end of the natural temperature range. These authors concluded that
874 high levels of certain HK cellular responses at 13°C corresponded to high
875 immunocompetence and that increased gene expression responses at higher
876 temperatures (correlating negatively with body condition) were indicative of
877 immunopathology and dysregulation. This interpretation for cellular responses is
878 partly consistent with our laboratory results. For example, under our present study
879 conditions, both *Saprolegnia* and *Schistocephalus* disease progression worsened as
880 the temperature rose (all other things being equal), although this could also relate to
881 the cold-biased expression of some innate immune pathways that we observed here.
882 On the other hand, we found that under natural circumstances (in mesocosms) high
883 expression of adaptive immunity genes (such as *ighm*) correlated with high
884 immunocompetence and also coincided with the warmest times of year.
885 Furthermore, in late summer (in the weeks following seasonal peaks in temperature)
886 we have not found fish exposed to natural temperature variation to undergo marked
887 reductions in condition (Hablützel *et al.*, 2016). Rather the genome-wide
888 transcriptomic signatures seen in wild fish at this time of year include adaptive
889 immune activity and also growth and development (Brown *et al.*, 2016), the latter
890 indicative of robust health. Taken together, these observations suggest that, within
891 the normal range of temperatures (although perhaps not at the more extreme
892 temperatures considered by Dittmar *et al.*), high immune gene expression does not
893 necessarily equate to dysregulation and may reflect effective resistance responses.

894 In conclusion, we generated a realistic mid-latitude climatic warming scenario in
895 outdoors mesocosms, incorporating precise temperature control. With this we
896 demonstrated significant perturbation of immunity and infectious disease progression
897 under modest incremental warming (+2°C) in a representative natural model CBV
898 (the three-spined stickleback). These perturbations included changes in both the

899 magnitude and phenology of disease that might be of practical importance in real-
900 world situations. Parallel laboratory experimental analyses confirmed that thermally-
901 driven responses of immunity and infectious disease progression were substantial.
902 When all else was equal, thermal effects were most strongly dependent on the
903 prevailing temperature (the latter, in the case of infection, here taken to encompass
904 temperature regimen post-invasion). Lagged thermal effects (preceding invasion, in
905 the case of infection) were less important. The contrasting responses to thermal
906 manipulation of our different infection systems confirm that increases in temperature
907 can worsen or ameliorate disease progression according to the specific thermal
908 biology of the host and pathogen. Thus, in an otherwise constant warming
909 environment, within-host outcomes would likely to play out in a system-specific way
910 in complex host-parasite communities, without necessarily increasing the overall
911 burden of disease. Most importantly, by combining our mesocosm observations with
912 experimentally-derived estimates of thermal effects, we show that, in a seasonal
913 natural system, thermal effects are superimposed upon substantial temporal
914 variation in immunocompetence. The latter is driven by non-thermal aspects of the
915 environment and, for *Saprolegnia*-mediated disease, its effect is at least as large as
916 that of thermal variation. Critically, thermal change is likely to indirectly affect the
917 non-thermal environmental drivers of immunocompetence, additional to its direct
918 effects on disease progression. Thus, projection of infection dynamics based on
919 experimentally-determined thermal effects alone is unlikely to be reliable, given the
920 size of non-thermal environmental effects on immunocompetence. In practical
921 management situations, the accuracy of such projections might be improved by
922 primarily considering prevailing (and not lagged) thermal effects and by incorporating
923 validated measures of immunocompetence (such as *ighm* expression in the case of
924 *Saprolegnia* here).

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