Short Title: Shifts in temperature and *Batrachochytrium*

Shifts in temperature influence how *Batrachochytrium dendrobatidis* infects amphibian larvae

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Author contribution statement

ARB, JRR, and TRR originally formulated the idea. PWB designed the experiment and developed the methodology. PWB performed the experiment. PWB and MDB performed the molecular analysis. PWB and TRR performed the statistical analyses. ARB, JRR, TRR, and DHO obtained funding. PWB wrote the manuscript and other authors provided editorial advice.

Abstract:

Many climate change models predict increases in mean temperature, and increases in frequency and magnitude of temperature fluctuations. These potential shifts may impact ectotherms in several ways, including how they are affected by disease. Shifts in temperature may especially affect amphibians, a group with populations that have been challenged by several pathogens. Because amphibian hosts invest more in immunity at warmer than cooler temperatures and parasites may acclimate to temperature shifts faster than hosts (creating lags in optimal host immunity), researchers have hypothesized that a temperature shift from cold-to-warm might result in increased amphibian sensitivity to pathogens, whereas a shift from warm-to-cold might result in decreased sensitivity. Support for components of this climate-variability based hypothesis have been provided by prior studies of the fungus *Batrachochytrium dendrobatidis* (Bd) that causes the disease chytridiomycosis in amphibians. We experimentally tested whether temperature shifts before Bd exposure alter susceptibility to Bd in the larval stage of two amphibian species – western toads (*Anaxyrus boreas*) and northern red legged frogs (*Rana aurora*). Both host species harbored elevated Bd infection intensities under constant cold (15° C) temperature in comparison to constant warm (20° C) temperature. Additionally, both species experienced an increase in Bd infection abundance when shifted to 20° C from 15° C, compared to a constant 20° C but they experienced a decrease in Bd when shifted to 15° C from 20° C, compared to a constant 15° C. These results are in contrast to prior studies of adult amphibians that found increased susceptibility to Bd infection after a temperature shift in either direction, highlighting the potential for species and stage differences in the temperature-dependence of chytridiomycosis.

Keywords: amphibian declines, *Batrachochytrium dendrobatidis*, chytridiomycosis, climate variability hypothesis, infectious disease, temperature, *Rana aurora*, *Anaxyrus boreas*

Introduction

Climate change represents one of the greatest challenges to biodiversity and conservation because it might compromise ecosystem functions worldwide. Most studies of climate-change induced effects on ecological communities emphasize the role of predicted changes to annual or seasonal mean temperatures or precipitation (Paaijmans, Read & Thomas 2009, Paaijmans *et al.* 2010). However, many climate change models predict increases in the frequency and magnitude of extreme weather events such as heat waves and droughts (Schar *et al.* 2004, Horton *et al.* 2016) leading to increases in temperature variability at shorter timescales (Easterling *et al.* 2000, Rummukainen 2012). These predicted climate-change induced increases in short-term temperature fluctuations can affect species interactions (Hoover, Knapp & Smith 2014, Teskey *et al.* 2015). Yet few studies have investigated how increases in temperature variability on these shorter timescales affect disease dynamics despite the likelihood that such variability might differentially affect hosts and pathogens (Ben-Horin, Lenihan & Lafferty 2012, Bannerman & Roitberg 2014, Raffel *et al.* 2015).

The aquatic chytrid fungal pathogen *Batrachochytrium dendrobatidis* (Bd) causes chytridiomycosis, an emerging infectious disease of amphibians (Longcore, Pessier & Nichols 1999). Bd is widespread globally (Liu, Rohr & Li 2013, Olson *et al.* 2013) and is

70 associated with worldwide amphibian population declines (Stuart *et al.* 2004, Skerratt *et*
71 *al.* 2007). Moreover, models based on IPCC climate projections predict that the range of
72 Bd will increase, thus potentially making more amphibian species at risk (Xie, Olson &
73 Blaustein 2016).

74 In larvae, Bd infection can cause host mortality in some species (Blaustein *et al.*
75 2005, Garner *et al.* 2009). However the infection is localized to keratinized larval
76 mouthparts, (Marantelli *et al.* 2004, McMahon & Rohr 2015) often resulting in sublethal
77 effects (Han, Bradley & Blaustein 2008, Buck *et al.* 2012, Gervasi *et al.* 2013).

78 Temperature is considered one of the most important environmental factors
79 driving chytridiomycosis (Forrest & Schlaepfer 2011, Voyles *et al.* 2017). Because
80 physiologies of both the host and pathogen are strongly influenced by environmental
81 temperature, climate change has been used to explain several major Bd outbreaks and
82 amphibian population declines, (reviewed in Li, Cohen & Rohr 2013, Rohr *et al.* 2013).
83 Yet, the host and pathogen are not expected to share a uniform response to a given
84 temperature (Brown *et al.* 2004, Rohr *et al.* 2013) and thermal responses measured in
85 constant-temperature artificial environments might not reflect organism responses in
86 more realistic variable-temperature environments. Providing evidence of the lack of a
87 uniform response between Bd and amphibians to temperature shifts, Rohr and Raffel
88 (2010) found a strong correlation between elevated month-to-month temperature
89 variability and Bd-associated amphibian population declines of *Atelopus* spp. across
90 Central and South America. Further support of the relationship between chytridiomycosis
91 and temperature variability has been provided by laboratory studies (Raffel *et al.* 2013,
92 Raffel *et al.* 2015).

93 The potential for temperature variability to increase disease severity in
94 amphibians was first postulated by Raffel *et al.* (2006) and has subsequently been
95 referred to as the "climate variability hypothesis" (Rohr & Raffel 2010). This hypothesis
96 posits that parasites acclimate to the new temperature more rapidly than their hosts,
97 leading to lags in host acclimation following a temperature shift that could make hosts
98 more susceptible to infection (Raffel *et al.* 2013). However, Raffel *et al.* (2006) also
99 pointed out potential complexities in acclimation of the ectotherm immune system that
100 might lead to alternative predictions. According to the "*lag effect*" hypothesis (Raffel *et*
101 *al*. 2006), changes in levels of temperature-dependent immune parameters might simply
102 lag behind environmental temperature shifts (Fig. 1) because it takes time to produce
103 necessary, or remove unnecessary, immune cells from the host. Thus, the "lag effect"
104 hypothesis predicts the opposite effect from the "climate variability hypothesis"
105 following a temperature decrease, at least on a short timescale. These mechanistic
106 hypotheses are not mutually exclusive, and it is unclear which effects might be more
107 important for a given host-parasite combination.
108 We tested the general prediction that an amphibian shifted to a new temperature
109 before Bd exposure would respond to infection differently than a host already acclimated
110 to the exposure temperature. We postulated that the direction of the effect would depend
111 upon the direction of the temperature shift, in accordance with the "lag effect" hypothesis
112 of Raffel *et al*. (2006). Given the differences in size between the host and the pathogen,
113 and associated physiological process rate differences, we assumed Bd would
114 physiologically respond to the temperature shift faster than the host, such that an
115 idealized host-immune response to Bd exposure would temporarily lag behind the

116 temperature shift. Thus, we predicted that a temperature shift from cold-to-warm would
117 result in an *increase* in susceptibility to Bd exposure, whereas a temperature shift from
118 warm-to-cold would result in a *decrease* in susceptibility to Bd exposure.
119
120 **Materials and Methods**
121 In a laboratory study under controlled conditions necessary for increased
122 precision and to allow for better replication, we examined how temperature shifts may
123 alter larval amphibian infection dynamics. We selected two species of amphibian hosts,
124 the northern red legged frog (*Rana aurora*) and the western toad (*Anaxyrus boreas*) as
125 adults of both species have been observed in the field with Bd infections (Pearl *et al.*
126 2007, Piovia-Scott *et al.* 2011) and both species are susceptible to chytridiomycosis (Han,
127 Bradley & Blaustein 2008, Gervasi *et al.* 2013). Amphibians were collected as eggs from
128 natural oviposition sites where Bd is not known to be endemic. Red legged frog eggs
129 were collected from a permanent pond located near Florence, Oregon, USA (Lincoln
130 County, elevation 12 m; latitude/longitude: 44.088/-124.123) in the Oregon Coast Range
131 on 11-Feb-2012. Western toad eggs were collected from Little Three Creeks Lake
132 (Deschutes County, elevation 2,000 m; latitude/longitude: 44.009/-121.643) in the
133 Cascade Range on 9-Jul-2011. Immediately after collection, eggs were transported to a
134 laboratory at Oregon State University where they were maintained at 14° C, under a 12-
135 12 photoperiod in 40-liter aquaria filled with dechlorinated water. Upon hatching, larvae
136 were maintained at a density of approximately 200 individuals per aquarium and fed *ad*
137 *libitum* a mixture of Tetramin fish food and ground alfalfa pellets (1:3 ratio by volume).
138 Water was changed every seven days. The 40-day trials for each species were not run

139 concurrently, but identical protocols were used for both species and both trials consisted
140 of individuals of identical larval stage (Gosner stage 26).

141 *Acclimation Period*

142 Independent trials for each host species began with a 20-day acclimation period
143 with 80 (Gosner stage 26) larvae randomly selected, and individually placed into 80
144 plastic 500-mL containers where they were housed for the duration of the acclimation
145 period and experiment. Each container was filled with 14° C dechlorinated water and
146 covered with a lid to help maintain water temperature and limit evaporation. Each
147 container had 2-mm diameter holes drilled between the water line and the lid to allow air
148 circulation into the container. Pairs of containers were then placed within 40 individual
149 temperature-controlled chambers that were set at 15° C to avoid cold-shocking the larvae.
150 Each temperature-controlled chamber was independently controlled via its own
151 thermostat and the interior measured approximately 37 cm deep x 21 cm wide x 13 cm in
152 height. Half of the 40 temperature-controlled chambers were then randomly selected to
153 begin the acclimation period at 20° C (warm treatment) and the other half were kept at
154 15° C (cold treatment). The placement of temperature chambers within the laboratory
155 was randomized, as was the placement of 500-mL containers within each temperature
156 chamber.

157 *Temperature Shifts*

158 On day 20 of the experiment, half of the temperature chambers in each of the two
159 acclimation temperatures (15° C and 20° C) were randomly selected to undergo a
160 temperature shift, either from 20° to 15° C or from 15° C to 20° C. The other half of the
161 temperature chambers underwent no shift in temperature. Thus, each of the temperature

chambers was subjected to one of four temperature treatments: a constant 15° C (cold) throughout the experiment; a constant 20° C (warm) throughout the experiment; a temperature shift from 15° C to 20° C (cold-to-warm); or a temperature shift from 20° C to 15°C (warm-to-cold).

Bd exposure

On day 24, one of the two 500-mL containers within each temperature-controlled chamber was randomly selected to undergo a Bd-exposure treatment and the other was selected as a control. Larvae in the Bd-exposure treatment were exposed to a single inoculate of Bd strain JEL 274, which was grown in pure culture on 1% tryptone agar in 10-cm diameter Petri dishes for 10 days. To harvest the zoospores, 10 plates were flushed with 15 mL of 15° C dechlorinated water and remained undisturbed for 10 minutes. The inoculum from each plate was then pooled in a beaker, quantified, and then diluted to 10,000 zoospores/mL. Individuals in the Bd-exposed treatments were exposed to 10 mL of inoculum transferred into the 500-mL container housing an individual larva. Control individuals were exposed to 10 mL of sham inoculum lacking the Bd culture (made from 1% tryptone sterile agar plates following the same methods). Thus, the individual larva underwent their exposure treatment on day 24, four days after the water temperature shift for chambers in the two temperature shift treatments.

During the 40-d trial, survival and metamorphic status were checked daily. Water for each 500-mL container within the temperature chambers was changed every 12 days and consisted of dechlorinated water of the same temperature (15° C and 20° C). Individuals that survived until the end of the trial (i.e., day 40) were euthanized in a 2% solution of MS-222, and then preserved in 95% ethanol. Individuals that reached

185 metamorphosis (Gosner stage 42: emergence of forelimbs) were euthanized, measured,
186 and preserved as previously described.

187 *Determining infection status*

188 We used quantitative polymerase chain reaction (qPCR) to determine infection
189 status and quantify Bd-infection intensity of all individuals in the Bd-exposure
190 treatments. Additionally, we investigated Bd-infection status in eight randomly selected
191 control individuals per species. To sample the individuals for Bd, we extracted whole
192 mouthparts of the larvae using sterile dissection scissors. We conducted qPCR using an
193 ABI PRISM 7500 sequencer (Applied Biosystems) according to the methods of Boyle *et*
194 *al*. (2004) except that we used 60 µL of Prepman Ultra (Applied Biosystems, Carlsbad,
195 California, USA), instead of the 40 µL in the DNA extraction.

196 *Statistical Analyses*

197 Each temperature-controlled chamber was subjected to one of four temperature
198 regimes consisting of a Bd-exposure temperature combined with a temperature shift
199 status (constant cold, constant warm, shifted to cold, and shifted to warm). Further, the
200 pairs of containers within each temperature-controlled chamber were subjected to one of
201 two exposure treatments (Bd exposed and Bd unexposed).

202 Survival was compared between temperature treatments for western toad larvae
203 with a Cox proportional hazards model using TIBCO Spotfire S+ version 8.1. The model
204 consisted of the main effects of the temperature treatment, temperature shift status
205 (constant versus shifted), and an interaction between the two variables. Due to losses of
206 western toad larvae prior to the application of the exposure treatment, we lacked the
207 power to statistically compare survival in western toad larvae between the Bd exposure

208 treatments

209 Bd infection abundance (Bd genomic equivalents) among temperature treatments
210 and between host species was analyzed using R version 3.11. We used a zero-inflated
211 negative-binomial generalized linear model (function 'zeroinf' in package 'pscl') as
212 described by Raffel *et al.* (2010). Our full model investigated the effects of all of the
213 explanatory variables including host species, exposure temperature, temperature shift
214 status, and all two- and three-way interactions on Bd abundance. Interpretation of this
215 analysis required further reduced models to investigate the effect of exposure temperature
216 and temperature shift for each species (species model) and the effect of temperature shift
217 for each Bd-exposure temperature and host species combination (Bd-exposure
218 temperature model).

219

220 **Results**

221 Survival differences were not detected between exposure temperatures (Cox, Z = -
222 1.099, $p = 0.27$) or temperature shift status (Cox, Z = -0.277, $p = 0.78$) in Bd-exposed
223 western toad larvae. We were unable to detect survival differences in red legged frog
224 larvae, as only one individual larva experienced mortality after application of the
225 exposure treatment (Table S1).

226

227 *Infection Abundance*

228 We detected a host species by temperature shift interaction ($\chi^2_1 = 3.83$, $p = 0.050$;
229 Table S2) and a Bd-exposure temperature by temperature shift interaction ($\chi^2_1 = 7.50$, $p =$

0.006; Table S2). We investigated these interactions with reduced models to investigate effects on Bd abundance at the levels of species and exposure temperature.

Red legged frog larvae had higher Bd abundance when they were exposed to infection at 15° C when compared to 20° C ($\chi^2_1 = 3.88$, $p = 0.049$; Fig. 2). The main effect of temperature shift was marginally significant in the reduced species model analysis ($\chi^2_1 = 3.50$, $p = 0.061$), but there was a significant effect of temperature shift for individuals exposed at 20° C in the reduced Bd-exposure model ($\chi^2_1 = 5.7$, $p = 0.017$), with individuals shifted from 15° C to 20° C having higher Bd abundance than red legged frog larvae experiencing constant 20° C (Fig. 2). In contrast, there was no evidence that a temperature shift influenced Bd infection when red legged frog larvae were exposed to Bd at 15° C ($\chi^2_1 = 0.6$, $p = 0.4$; Fig. 2). There was no statistically significant interaction between exposure temperature and temperature shift for red legged frog larvae ($\chi^2_1 = 2.4$, $p = 0.13$).

We detected an interactive effect of exposure temperature and temperature shift on Bd abundance in western toad larvae ($\chi^2_1 = 5.2$, $p = 0.023$). This was driven by elevated Bd abundance in individuals under the constant 15° C temperature when compared to individuals that experienced a temperature shift from 20° to 15° C, but no evidence of an effect of shifting temperature from 15° C to 20° C (Fig. 2). There were no main effects of exposure temperature ($\chi^2_1 = 0.50$, $p = 0.5$) or temperature shift ($\chi^2_1 < 0.01$, $p = 0.9$) on Bd abundance in western toad larvae. Further, when investigating the exposure temperatures individually in the reduced Bd-exposure model, there was no evidence that a temperature shift influenced Bd infection in western toad larvae after exposure to Bd at 15° C ($\chi^2_1 = 3.4$, $p = 0.066$) or 20° C ($\chi^2_1 = 2.5$, $p = 0.11$).

We failed to find evidence that the two host species differed in response to exposure to the pathogen, leading us to conclude that general patterns for both species were similar (Fig. 2).

A number of western toad individuals died or metamorphosed before the end of the experiment (Table S2) but the model for Bd abundance on western toads was not significantly improved by adding either a variable coding whether individuals were near metamorphosis when sampled ($\chi^2_1 = 4.00$, $p = 0.150$) or a covariate indicating the sampling date ($\chi^2_1 = 3.33$, $p = 0.068$). Furthermore, neither variable qualitatively changed the contribution of exposure temperature or temperature shift status to the model. Therefore, we omitted both covariates from the final model for western toads.

Discussion

Numerous climate change models predict increases in annual or seasonal mean temperatures in many locations (IPCC 2007). These models often also predict elevated chances of extreme weather events occurring at, and over, much shorter timescales (Rummukainen 2012, Horton *et al*. 2016). Temperature shifts that may be associated with the onsets and conclusions of these weather events have the potential to affect alter species interactions – including host-pathogen interactions (Rohr & Raffel 2010, Ben-Horin, Lenihan & Lafferty 2012, Bannerman & Roitberg 2014).

Our results suggest that Bd infection dynamics in larval amphibians can be affected by a shift in water temperature before host exposure to the pathogen, and that the direction of temperature shift determines the outcome of Bd exposure. Importantly, we detected the effects of temperature shifts despite the host having a four-day head start on

276 acclimating to the Bd exposure temperature relative to the pathogen. This suggests that
277 we are likely underestimating the strength of these effects and that their magnitudes
278 might have been larger if the host and pathogen experienced the shifts concurrently, as
279 which would most likely be expected in the field.

280 Amphibian species do not all respond similarly to a given Bd exposure (Searle *et*
281 *al*. 2011, Gervasi *et al.* 2017). For some susceptible host species, temperature-shift
282 induced changes in Bd abundance might alter the outcome of infection by either pushing
283 *Bd* abundance over or under a tolerance threshold. Such changes in relation to pathogen
284 abundance and pathogen tolerance may result in altering the strength of negative effects
285 of Bd infection.

286 Our results were consistent with predictions of the "lag effect" hypothesis (Raffel
287 *et al.* 2006, Rohr & Raffel 2010), and were generally consistent with previous studies
288 showing that a shift in temperature influences Bd infection in postmetamorphic
289 amphibians (Raffel *et al.* 2013, Raffel *et al.* 2015). In particular, our finding of decreased
290 resistance to infection following a temperature increase (relative to warm-acclimated
291 individuals) mirrored a laboratory study of post-metamorphic red-spotted newts
292 (*Notophthalmus viridescens*), where juvenile newts exhibited decreased Bd resistance
293 following a shift from 15° C to 25° C (Raffel *et al.* 2015). These findings of fluctuating
294 temperature effects on Bd infection across amphibian taxonomic groups and life-stages
295 suggest that effects of temperature shifts and Bd-related chytridiomycosis susceptibility
296 might be widespread within amphibians. However, our finding of increased resistance to
297 Bd infection following a temperature decrease (relative to cold-acclimated individuals)
298 was opposite the pattern observed in red-spotted newts and Cuban treefrogs (Raffel *et al.*

299 2013, Raffel *et al.* 2015) These contrasting results suggests that there are important
300 among-taxa or among-stage differences in the underlying mechanisms driving the effects
301 of temperature fluctuation on Bd infection; whereas our results in pre-metamorphic life-
302 stage of western toads and red legged frogs are consistent with the "lag effect"
303 hypothesis, results of similar studies investigating post-metamorphic red-spotted newts
304 and Cuban treefrogs support the "climate variability hypothesis."

305 Higher Bd abundances were observed for both host species under the constant
306 cold temperature treatment compared to the constant warm temperature treatment. These
307 results are consistent with previous experiments that showed increased Bd abundance
308 (Raffel *et al*. 2015) and Bd-induced mortality (Kilpatrick, Briggs & Daszak 2010, Raffel
309 *et al.* 2015) associated with lower temperatures. This is despite Bd growing best in
310 culture at about 23° C, which is much closer to the warm than cold temperature
311 treatments in this experiment (Piotrowski, Annis & Longcore 2004, Woodhams *et al.*
312 2008). This might be because the larval immune response to Bd infection increases with
313 increasing temperatures at a faster rate than the infectivity or growth rate of Bd (Raffel *et*
314 *al*. 2013), or alternatively because of the differences between the growth rate of Bd in
315 culture compared to the growth rate on host tissue (Venesky *et al*. 2013).

316 Alternatively, differences in Bd abundance between the two constant temperature
317 treatments might be due to temperature effects on the pathogen rather than the host
318 (Woodhams *et al.* 2008, Voyles *et al.* 2012). The Bd was cultured at 15° C; it is possible
319 that the temperature shift experienced by the pathogen in the warm exposure treatment
320 caused the depressed Bd abundances observed in both host species compared to the
321 elevated Bd abundance in the cold exposure temperature treatment. A decrease in

322 temperature may cause an increase in the number of Bd zoospores released from
323 zoosporangia (Woodhams *et al*. 2008), however the effect of a similar increase in
324 temperature on Bd physiology is unclear.

325 In conclusion, our results provide additional evidence for climate variability
326 affecting Bd infection in amphibians but suggest important among-taxa differences in the
327 directionality of these effects. Our study highlights the complexity that temperature plays
328 in determining the outcome of Bd-amphibian interactions and the role that a fluctuating
329 temperature might play in altering these interactions. Furthermore, this study increases
330 the diversity of amphibian species and stages that have been shown to exhibit thermal
331 acclimation effects on disease, and the broad generality of this pattern across four
332 disparate taxa suggests that fluctuating-temperature effects on amphibian infection may
333 be widespread.

334

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351

352 Conflict of Interest: The authors declare that they have no conflict of interest.

353

354 **Supporting Information**

355 Additional supporting information may be found in electronic supplementary
356 material for this article.

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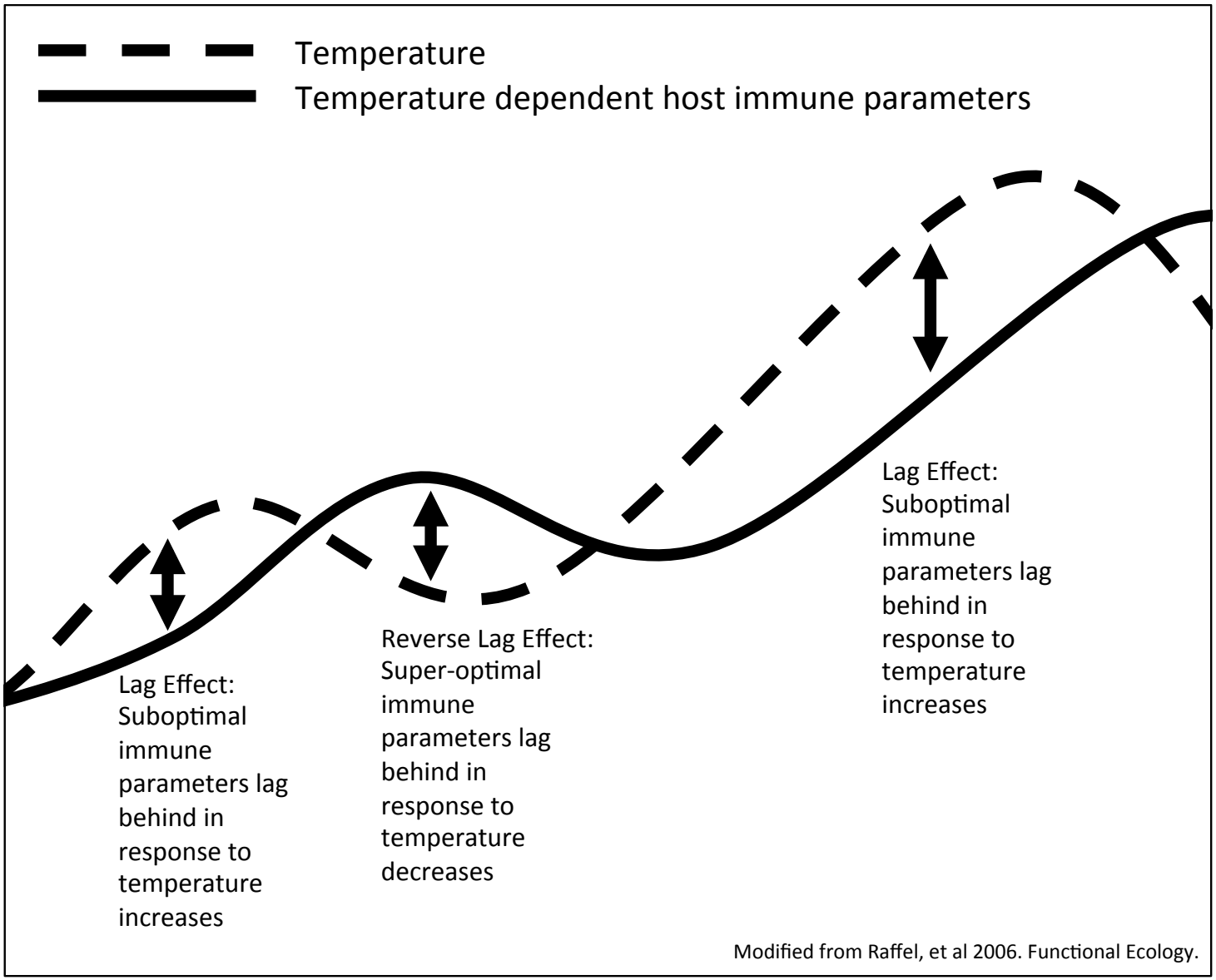
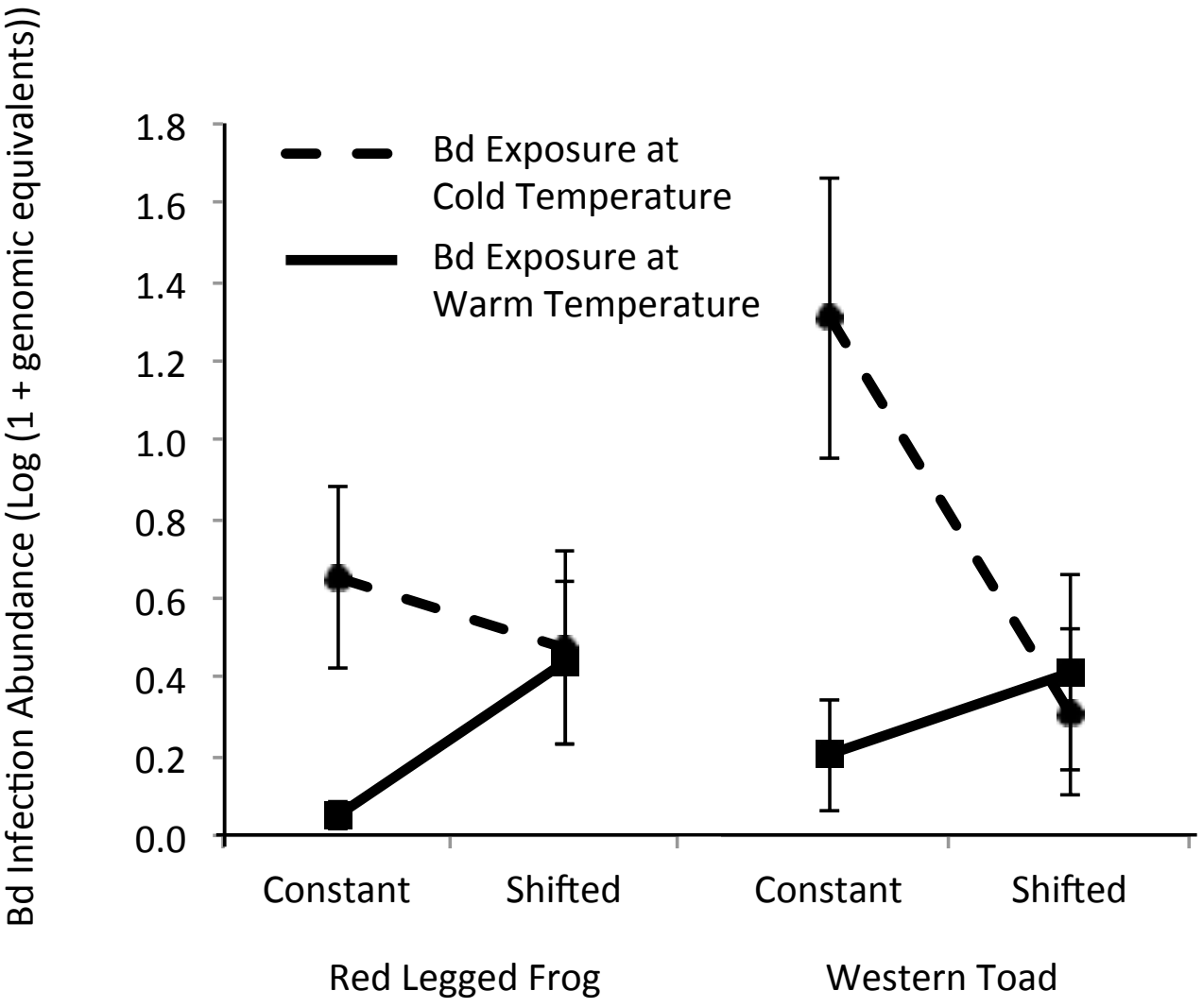
497

498

499 **Fig 1.** Hypothesized lag effect showing the relationship between fluctuating
500 temperatures (over days to weeks) and the optimal levels of a hypothetical
501 temperature-dependent host immune parameter. The immune parameter follows and
502 lags behind temperature changes – resulting in periods of a compromised immune
503 status after a temperature increase, and resulting in an over-active (or unnecessarily
504 costly) immune status after a temperature decrease. Modified from Raffel *et al*.
505 (2006).
506
507 **Fig 2.** Mean *Batrachochytrium dendrobatidis* (Bd) infection abundance (± SE)
508 measured at death, or at euthanasia 16-days after Bd exposure, in both western toad
509 (*Anaxyrus boreas*) larvae and red legged frog (*Rana aurora*) larvae from Oregon,
510 USA, and between the two temperatures at the time of Bd-exposure (cold [15° C]
511 versus warm [20° C]) and between larvae having experienced either a constant or
512 shifted temperature. Bd infection abundance is quantified as the log (1 + Bd genomic
513 equivalents) per excised larval mouthparts of all individuals exposed to the pathogen.
514


Temperature
Temperature dependent host immune parameters
Lag Effect: Suboptimal immune parameters lag behind in response to temperature increases
Reverse Lag Effect: Super-optimal immune parameters lag behind in response to temperature decreases
Lag Effect: Suboptimal immune parameters lag behind in response to temperature increases
Modified from Raffel, et al 2006. Functional Ecology.
Time in Days


Bd Infection Abundance (Log (1 + genomic equivalents))
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Bd Exposure at Cold Temperature
Bd Exposure at Warm Temperature
Constant
Shifted
Constant
Shifted
Red Legged Frog
Western Toad