

# Critical windows of disease risk: amphibian pathology driven by developmental changes in host resistance and tolerance

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## Summary

**1.** An emerging framework in animal disease ecology seeks to ‘decompose’ a host’s response to disease into resistance, or its ability to resist infection following exposure, and tolerance, or its ability to limit the damage associated with infection. How these processes vary over the life history of a host, however, and whether developmental changes in resistance and tolerance account for ‘critical windows’ of disease vulnerability remain open questions.

**2.** Critical developmental windows are particularly important for infections that alter host development. Recently, increased observations of amphibians with severe limb malformations have stimulated debate over the causes responsible and whether malformation types can be used to infer the agent responsible. The trematode parasite *Ribeiroia ondatrae*, for example, is often implicated in accounts of extra-legged frogs, but is believed to be unimportant in explaining missing legged animals. Here, we test the influence of host developmental stage, from eggs to post-metamorphosis, on the risk of mortality and the types of malformations produced in Pacific chorus frogs (*Pseudacris regilla*) following exposure to trematode infection.

**3.** Consistent with a critical window of vulnerability, host mortality and malformations were greatest among animals exposed during pre-limb and early limb development (15–90%) and decreased to < 5% with progressive development. Early stage animals also exhibited a higher frequency of missing limbs, whereas extra limbs and limb elements developed predominantly among tadpoles exposed after limb development was initiated. Hosts infected later in limb development were normal or exhibited only minor outgrowths and abnormal skin webbings.

**4.** Increases in host tolerance rather than host resistance largely explained the observed changes in pathology. Prior to host metamorphosis, parasites exhibited comparable success invading host tissue, but the amount of resulting damage differed significantly as a function of host size and developmental stage. Following metamorphosis hosts were significantly more resistant to infections, however.

**5.** These findings highlight the importance of critical developmental windows for infectious diseases and underscore the role of developmental changes in host tolerance in controlling this process. Forecasted changes in climate, for example, have enormous potential to influence both the timing and intensity of host–parasite interactions in nature.

**Key-words:** amphibian decline, emerging disease, infectious disease, malformations, parasite, *Ribeiroia*, trematode

## Introduction

Disease is often the product of complex interactions among hosts, pathogens and the environment in which they are embedded (e.g. Plowright *et al.* 2008; Skerratt, Garner &

Hyatt 2010). While well accepted in principle, the importance of such interactions is rarely quantified. Changes in host age or condition, for example, can significantly alter patterns of infection and disease. In toxicology and teratology, which focus on non-infectious diseases, subtle changes in host developmental stage can lead to profoundly different forms of pathology, as illustrated tragically through

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discovery of foetal alcohol syndrome, DES toxicity, thalidomide teratogenesis and mercury poisoning (Mittendorf 1995; Dally 1997; Tabin 1998; Astley *et al.* 1999; Tchounwou *et al.* 2003). Such developmental sensitivity is often referred to as a 'critical window' in development, during which exposure to a toxin (or lack of an essential nutrient) causes impairment (Bunn *et al.* 2001; Kalter 2003; Pryor *et al.* 2000). Similar patterns have been observed for infectious diseases, in which susceptibility to infection or the risk of pathology vary with host age or prior exposure. Infections of very young or very old hosts, for instance, are often more likely to be associated with human illness and pathology (Baird 1998; Thompson *et al.* 2004; Goodwin, Viboud & Simonsen 2006). The occurrence of such age-dependent pathology creates complexity in understanding the aetiology of diseases in both humans and wildlife.

Hosts of different ages or developmental stages can vary in disease risk because of differences in either susceptibility to infection or the likelihood of developing pathology following infection. An emerging framework in disease ecology seeks to mechanistically understand the disease process by distinguishing between these two processes, which can be classified as 'resistance', or the ability of a host to limit infection following exposure, and 'tolerance', or the ability of a host to limit pathology following infection (Boots 2008; Read, Graham & Råberg 2008; Råberg, Graham & Read 2009; Rohr, Raffel & Hall 2010). This framework and its statistical application stem from nearly a century of use in plant disease research (e.g. Kover & Schaal 2002). While often confused in the animal disease literature, resistance and tolerance have very different implications for the ecology and evolution of a pathogen. Higher host resistance, for instance, places a greater selective pressure on pathogens to increase virulence, whereas tolerance may have a negative or neutral effect on virulence (Råberg, Graham & Read 2009). Similarly, while a resistant host may act as a 'decoy' or 'dilution' host for multi-host pathogens, even helping to reduce disease risk (see Johnson & Thieltges 2010), tolerant hosts may be more likely to act as disease reservoirs, maintaining infection in a community even as sensitive (less tolerant) host species decline (Lloyd-Smith *et al.* 2005).

Critical windows of development that influence properties of host resistance and tolerance may have particular relevance for infections that induce morphological abnormalities (MacLachlan, Conley & Kennedy 2000). In North America, for example, infection by the trematode *Ribeiroia ondatrae* causes larval amphibians to develop severe limb deformities, including missing, extra and misshapen limbs (Sessions & Ruth 1990; Johnson *et al.* 1999, 2001; Stopper *et al.* 2002; Johnson & Hartson 2009). Previous work has established that (i) infection dosage influences the risk of mortality and malformations; (ii) pathology varies among host species; and (iii) the timing of exposure can influence the probability of death and deformity (see Johnson *et al.* 2010). Schotthoefer *et al.* (2003b) reported that tadpoles exposed at the pre-limb bud stage were more likely to exhibit mortality, whereas those infected during early limb development had higher survival

rates but exhibited developmental malformations. Bowerman & Johnson (2003) suggested that variation among amphibian species' phenology and the timing of infection may help to explain patterns of malformation in nature and why some species are more likely to exhibit high frequencies (e.g. > 20%) of developmental abnormalities.

However, the mechanisms driving such observations and whether stage-dependent pathology owes to changes in host resistance, host tolerance or both remains largely unexplored. Moreover, a pressing problem in pursuing this question involves how, exactly, resistance and tolerance are measured. A common strategy in infection experiments is to measure parasite load either at death (among animals suffering mortality) or at the end of the experiment (among survivors). For macroparasites that do not reproduce within a host, however, parasite recovery decreases with time post-exposure as a result of host immunity and/or parasite mortality (i.e. clearance rate) (Telfer *et al.* 2008; Holland 2009; Johnson & Hartson 2009). This creates a confounding problem: do hosts that die have more parasites because of greater infection success (which caused their death) or because they were examined for infection significantly earlier than were survivors? Given that parasites can also delay host development (see Johnson & Buller *in press*), thereby providing additional time for parasite clearance to occur, experiments need to recognize that host resistance is a time-dependent process and needs to be measured at a single point in time or analyzed with time post-exposure as a covariate.

Here, we use the *Ribeiroia*-amphibian system as a model for exploring whether changes in host resistance or host tolerance explain stage-dependent variation in pathology. By exposing amphibians to *Ribeiroia* across a wide range of developmental stages, ranging from eggs to post-metamorphic frogs, our goals were to (i) isolate the 'critical window' of development during which amphibians experience mortality and malformations; (ii) evaluate how the types of malformations induced vary with the developmental stage of exposure; and (iii) test whether observed changes owe to shifts in host tolerance, host resistance or both. Importantly, because of the potential loss of parasites with increasing time post-exposure, we measured parasite recovery during a narrow window of time (10 days), rather than following metamorphosis. If observed patterns are the result of developmental changes in resistance alone, we would expect infection intensity (number of recovered parasites) to predict pathology with no additional contributions from stage of exposure. However, if the occurrence of a critical developmental window were due to changes in host tolerance, we would expect a significant interaction between infection and stage of exposure in explaining mortality, such that the slope of the relationship varies among groups (e.g. Råberg, Graham & Read 2009). Because of the large number of pathogens with stage- or age-dependent effects, understanding the relative roles of resistance and tolerance in explaining developmental heterogeneity in disease risk has broad relevance for human and wildlife diseases alike. Persistent controversies over the types of amphibian malformations induced by different agents further underscore

the applied contributions of this study in the continued investigation of amphibian malformations (e.g. Ballengee & Sessions 2009; Johnson & Bowerman 2010; Skelly & Benard 2010).

## Materials and methods

### STUDY SYSTEM

*Ribeiroia ondatrae* is a digenetic trematode that requires three hosts to complete its life cycle. Asexual reproduction occurs in freshwater snails of the family Planorbidae, leading to release of free-swimming cercariae that are infectious to larval amphibians and some fishes (Beaver 1939; Johnson *et al.* 2004). Cercariae lose their tails and form encysted metacercariae in these second intermediate hosts. Because cysts often form around the developing limbs of larval amphibians, they can cause developmental disruption and improper limb formation (Stopper *et al.* 2002). Such malformations are hypothesized to increase the probability that infected frogs are consumed by the definitive host, which is most often a bird (Johnson *et al.* 2004; Rohr, Raffel & Sessions 2009). Following consumption of an infected frog or fish, birds become infected with the adult stage of the parasite, which reproduces sexually within the host and releases eggs that are distributed across the landscape along with the host's faeces (Johnson & McKenzie 2008).

### EXPERIMENTAL EXPOSURES

We collected egg masses of *Pseudacris regilla* from Lake Penhollow, Oregon (43-8907, -121-4111; 1297 m), and shipped them to Colorado where they were acclimated to commercial spring water. We used *P. regilla* owing to its sensitivity to *Ribeiroia* infection and its tendency to exhibit a wide range of parasite-induced malformations following parasite exposure, ranging from completely missing limbs to multiple extra limbs (Johnson *et al.* 1999, 2002). Upon reaching the appropriate developmental stage, individual tadpoles (or egg masses) were transferred into 1.0 L containers and maintained at 23 °C. We exposed *P. regilla* to *Ribeiroia* cercariae at each of the following six distinct developmental periods: egg masses (Gosner 1960; stage 15–17;  $n = 15$ ), pre-limb growth [stages 23 ( $n = 19$ ) and 24 ( $n = 35$ )], early limb development [stages 26 ( $n = 67$ ), 28 ( $n = 53$ ) and 30 ( $n = 35$ )], late limb growth [stage 32 ( $n = 20$ ) and 34 ( $n = 20$ )], post-limb development [stage 37 ( $n = 20$ )] and following metamorphosis [stage 47 ( $n = 20$ )]. To provide a control treatment for mortality and malformation comparisons, 35 tadpoles (stage 26) were sham-exposed to water from an uninfected snail and raised to metamorphosis. All experimental tadpoles (Gosner 23–37) were exposed to 10 cercariae in a volume of 1.0 L. This dosage was selected based on extensive previous experiments to provide an intermediate yet realistic level of infection, capable of inducing pathology but unlikely to cause complete mortality (Johnson *et al.* 1999, 2001). However, to test whether late-stage tadpoles were, in fact, no longer vulnerable to parasite-induced pathology, we also exposed 20 late-stage tadpoles (Gosner stage 32) to 30 *Ribeiroia* cercariae as above.

Exposure of amphibian eggs and post-metamorphic frogs required a slightly different approach relative to tadpoles. For *P. regilla* eggs, which occur in groups of 15–50, we exposed three masses to 50 cercariae in a volume of 100 mL with the primary goal of assessing whether cercariae could detect and penetrate unhatched larvae. For post-metamorphic frogs, which spend most of their time out of water, we exposed individual frogs to cercariae in 20 mL scintillation vials filled

with 7 mL of water. Vials were turned horizontally and rotated periodically to ensure frogs were in constant contact with water but would not drown. We used a higher dosage of cercariae (20) for this group because we expected frogs would be insensitive to infection and wanted to maximize the likelihood of detecting encysted parasites.

We obtained cercariae of *R. ondatrae* from *Helisoma trivolvis* snails collected at field sites in California and Oregon. Individual snails were isolated into 50 mL centrifuge tubes, maintained in the dark from 17:00 to 21:00 hrs, and then checked for the presence of free-swimming cercariae. After identifying cercariae as *R. ondatrae* (see Johnson *et al.* 2004), we pooled parasites from individual snails and used a stereo dissecting scope to administer cercariae to tadpoles with a glass pipette (see Johnson & Hartson 2009). Ten cercariae were added to each tadpole's container and allowed to infect the animal. Although tadpoles varied considerably in size as a function of developmental stage, cercariae are highly effective at locating amphibian hosts within small volumes of water (Kiesecker 2002; Johnson & Hartson 2009).

Amphibians were monitored for mortality over a period of 10 days following exposures. Animals that died were necropsied immediately to quantify infection. Tadpoles were fed a diet of 1 : 1 Tetramin to *Spirulina* administered *ad libitum* while water and containers were replaced twice weekly. A subset of animals ( $n = \sim 10$ ) from each experimental group were euthanized after 48 h and subsequently necropsied to minimize the confounding effects of time since exposure in determining parasite recovery. Following the 10-day period, surviving animals were combined by treatment into 33 L containers ( $n = 20$  tadpoles per container) and raised to metamorphosis (Gosner 1960; stage 42). We described malformations in metamorphic frogs using a dissecting scope following the methods and terminology of Johnson *et al.* (2001). This approach assumes that a frog's malformation status (yes or no) is determined by parasite exposure alone and will be independent of being raised alongside other animals, which is reasonable given the rarity of severe limb malformations under normal rearing conditions. This same assumption cannot be made for growth and mortality, however, and as such we did not include time to metamorphosis or any mortality data beyond the 10 days following exposure in our analyses (as these could be influenced by co-housed animals). A subset of metamorphosing frogs was necropsied to quantify *Ribeiroia* metacercariae as above.

### ANALYSIS

We analyzed the effect of *Ribeiroia* exposure on host mortality and malformation status using logistic regression with Firth's correction in cases for which independent variables were overpredictive (Heinze & Schemper 2002). Survival was assessed only during the 10-day period following exposure, which is the general time frame during which parasite-induced mortality occurs (Johnson & Hartson 2009; Rohr, Raffel & Hall 2010), whereas malformations were assessed among animals surviving to metamorphosis. We evaluated the effects of developmental stage on parasite recovery only for animals necropsied within 10 days of exposure to minimize the effects of time since exposure on estimates of resistance. This included tadpoles that had died and those that were euthanized. Host resistance was estimated as the inverse proportion of successful parasites recovered in a host following exposure and we used linear regression to evaluate the effects of host stage and days-since-exposure (as a covariate) on this response measure. We used the proportion of parasites recovered (rather than the raw number of metacercariae) because metamorphic frogs were exposed to 20 rather than the 10 cercariae administered to tadpoles. However, after excluding metamorphic frogs, we also conducted the analysis on the raw numbers of *Ribeiroia* recovered using a

generalized linear model with a Poisson distribution and log-link function with appropriate tests for overdispersion (see Bolker *et al.* 2008; Zuur *et al.* 2009).

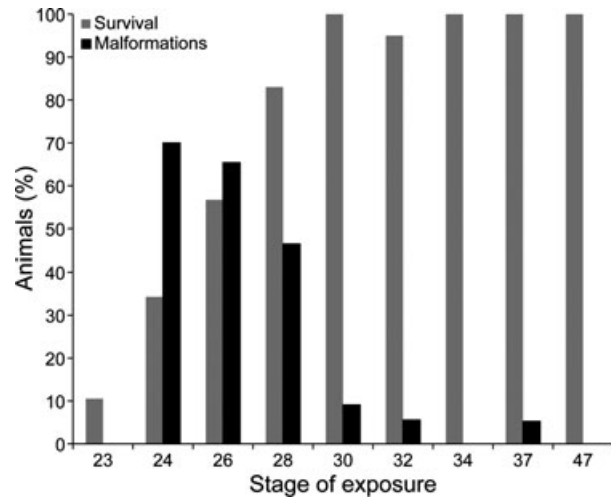
Following Råberg, Graham & Read (2009), who summarized the statistical approach used in the plant disease literature, we estimated tolerance as the slope of the relationship between parasite burden and host fitness (here as survival). An effect of tolerance is detected as a significant interaction term between parasite burden and group (here as developmental stage) in predicting host fitness. We therefore tested for tolerance by conducting a logistic regression analysis to predict 10-day mortality (yes or no), with parasites recovered, host developmental stage, and the stage-by-infection interaction term as independent variables. For some measures of fitness, 'general vigour' or the fitness in the absence of infection can vary by host species or genotype. We assumed that survival in the absence of infection was constant (i.e. 100%) across developmental stages, which is supported by the following observations: (i) all tadpoles were of the same species and genotype; (ii) unexposed animals at early development stages (26) had 100% survival; and (iii) even exposed animals at later stages had 95–100% survival.

## Results

### SURVIVAL AND MALFORMATIONS

Exposure to 10 *Ribeiroia* cercariae caused a significant increase in 10-day host mortality relative to unexposed tadpoles ( $\chi^2 = 34.73$ , d.f. = 1,  $P < 0.0001$ ;  $n = 105$ ). While all unexposed tadpoles survived, 43% of tadpoles infected at stage 26 died within 10 days of exposure, consistent with previous work on the lethality of *Ribeiroia* (Johnson *et al.* 1999). However, parasite-induced pathology depended strongly on the developmental stage of the host at the time of exposure. Mortality was greatest among early staged tadpoles and decreased monotonically with stage of exposure ( $\chi^2 = 113.75$ , d.f. = 1,  $R^2 = 0.49$ ,  $P < 0.0001$ ; stage coefficient =  $-0.66$ , Wald = 52.33, odds ratio (OR) = 0.515,  $n = 269$ ; Fig. 1). Whereas nearly 90% of tadpoles exposed at stage 23 died within 10 days, mortality fell to 0% by stage 30 and remained at  $< 5\%$  for all later-staged animals (one tadpole exposed at stage 30 died). No cercariae penetrated *P. regilla* egg masses and all exposed eggs hatched successfully.

The occurrence and form of developmental malformations in metamorphosing *P. regilla* also depended on the timing of exposure, although the window was less narrow than that observed for survival. Because of low survival among exposed tadpoles in the earliest developmental stages (23–24), we pooled metamorphic animals from these stages for comparison with later stages that exhibited higher survival. As with mortality, malformation frequency was greatest among tadpoles exposed at or before limb development (Gosner 1960; stages 23–28), with steadily declining malformation levels thereafter ( $\chi^2 = 50.95$ , d.f. = 1,  $R^2 = 0.39$ ,  $P < 0.0001$ ; stage coefficient =  $-0.45$ , Wald = 31.12, OR = 0.64,  $n = 166$ ; Fig. 1). However, low frequencies of relatively minor malformations were observed even in animals exposed at stage 30 (9.1%), stage 32 (5.6%) and stage 37 (5.3%). Stage 32 tadpoles exposed to 30 cercariae rather than 10 did not



**Fig. 1.** Survival and malformations in larval amphibians (*Pseudacris regilla*) exposed to *Ribeiroia ondatrae* infection at different stages of development. Grey bars reflect the percentage of animals surviving for 10 days or longer following an exposure to 10 parasite cercariae. Black bars reflect the percentage of animals with limb malformations among individuals that survived to metamorphosis. Stage of exposure indicates the developmental stage (Gosner 1960) during which the amphibians were exposed to infection.

exhibit significantly more malformations (6.3% vs. 5.6%,  $\chi^2 = 0.27$ , d.f. = 1,  $P = 0.869$ ). Only one of the unexposed animals exhibited an abnormality at metamorphosis (a missing eye), relative to abnormalities in 65.4% of animals exposed to *Ribeiroia* ( $\chi^2 = 37.25$ , d.f. = 1,  $P < 0.0001$ ; *Ribeiroia* coefficient = 0.383,  $n = 64$ ).

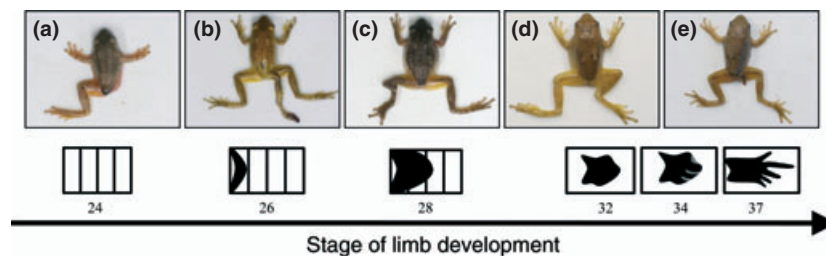
Qualitatively, the types and severity of malformations also changed as a function of stage of exposure (Table 1; Fig. 2). Among tadpoles exposed at stage 24 (pre-limb development), completely missing limb abnormalities predominated (four of five observed abnormalities). For animals exposed at stage 26 (early limb development), bony triangles (43.8%) and extra limbs or projections (37.5%) were the most common malformations ( $n = 32$  abnormalities). Skin webbings or cutaneous fusion occurred only among tadpoles exposed at stage 28 (30%,  $n = 20$  abnormalities) or later (Table 1; Fig. 2). The few abnormalities observed in animals exposed after stage 30 involved minor skin webbings ( $n = 1$ ) or femoral projections ( $n = 4$ ), which are unarticulated, digit-like outgrowths with variable levels of ossification (Fig. 2; Johnson *et al.* 1999).

### PARASITE EXPOSURE AND HOST RESISTANCE

To evaluate the effects of exposure stage on parasite recovery, which is the inverse of host resistance, we compared the proportion of *Ribeiroia* metacercariae (arcsin-square root transformed) isolated from tadpoles examined within 10 days of exposure. Parasite recovery decreased significantly as a function of host developmental stage and days post-exposure ( $R^2 = 0.53$ ,  $F_{2,164} = 90.61$ ,  $P < 0.0001$ ; days post-exposure

**Table 1.** Composition of limb abnormalities observed in metamorphosing amphibians (*Pseudacris regilla*) exposed to *Ribeiroia ondatrae* infection. The occurrence and relative abundance of different abnormalities varied as a function of the developmental stage in which amphibians were exposed to parasite infection, including pre-limb growth (Gosner 1960; stage 24), early limb development (stages 26–30), late limb development (stages 32–34) and post-limb development (stage 37). Control animals that were not exposed to parasites did not develop abnormalities

Abnormality type	Pre-limb		Early limb		Late limb		Post-limb
	Stage 24 <i>N</i> = 10	Stage 26 <i>N</i> = 26	Stage 28 <i>N</i> = 28	Stage 30 <i>N</i> = 11	Stage 32 <i>N</i> = 18	Stage 34 <i>N</i> = 20	Stage 37 <i>N</i> = 19
Missing limb	80% (5)	6.3% (2)	0	0	0	0	0
Partially missing limb	0	3.1% (1)	0	0	0	0	0
Missing digit(s)	0	6.3% (2)	0	0	0	0	0
Bony triangle	0	43.8% (14)	10% (2)	0	0	0	0
Extra limb	0	15.6% (5)	10% (2)	25% (1)	0	0	0
Extra foot	0	3.1% (1)	15% (3)	0	0	0	0
Extra digit	0	0	0	0	0	0	0
Skin webbing	0	0	30% (6)	50% (2)	25% (1)	0	0
Femoral projection	0	18.8% (6)	20% (4)	25% (1)	75% (3)	0	100% (1)
Micromelia	0	0	0	0	0	0	0
Other	1 (20%)	0	15% (3)	0	0	0	0
Total abnormalities	6	32	20	4	4	0	1



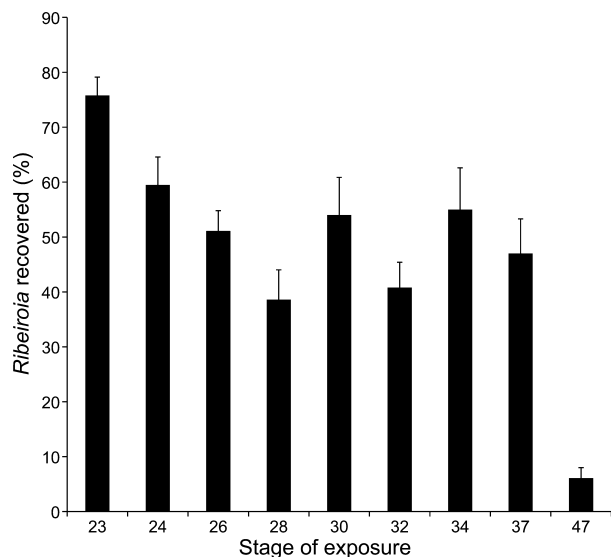
**Fig. 2.** Representative types of limb malformations induced in frogs exposed to *Ribeiroia* infection at different stages of development. Stage of exposure follows Gosner (1960), including pre-limb development (stage 24), early limb development (stages 26–28), late limb development (stages 32–34) and post-limb development (stage 37). Induced malformations include (a) missing limb, (b) extra limb, (c) bony triangle, (d) skin webbing and (e) femoral projection.

coefficient =  $-0.097$ ,  $P < 0.0001$ ; stage of exposure coefficient =  $-0.01759$ ,  $P < 0.0001$ ). However, this pattern was driven predominantly by the low parasite recovery in metamorphic frogs (Fig. 3). Despite being exposed to twice as many parasites, fewer than 50% of frogs exposed after metamorphosis (stage 47) became infected and the average infection  $\pm 1$  SE was  $1.33 \pm 0.38$  metacercariae per frog; in contrast, 98% of *P. regilla* exposed as larvae supported evident infection, with an average of  $5.30 \pm 0.21$  metacercariae per animal. If metamorphic frogs were excluded from the analysis, stage of exposure was no longer a significant predictor of *Ribeiroia* recovery ( $R^2 = 0.31$ ,  $F_{2,143} = 31.93$ ,  $P < 0.0001$ ; days post-exposure coefficient =  $-0.098$ ,  $P < 0.0001$ ; stage of exposure,  $P = 0.08$ ). Results were identical if we used a Generalized Linear Model with a Poisson distribution to predict the number of *Ribeiroia* recovered for stages exposed to 10 cercariae (Gosner stages 23–37) ( $\chi^2 = 18.88$ , d.f. = 1,  $P < 0.0001$ ; stage of exposure coefficient =  $-0.047$ ,  $P < 0.0001$ ). Interestingly, and as predicted, if we examined only animals exposed as larvae that survived to metamorphosis, stage of exposure had a positive effect on parasite recovery ( $R^2 = 0.07$ ,  $F_{1,94} = 7.67$ ,  $P = 0.0067$ ;

stage of exposure coefficient =  $0.0189$ ,  $P = 0.0067$ ;  $n = 96$ ). This owed to the fact that tadpoles exposed later in development had less time to clear their parasites prior to metamorphosis.

#### HOST TOLERANCE

Following the approach to estimating tolerance from the plant disease literature (see Råberg, Graham & Read 2009), we evaluated the effects of parasite recovery, stage of exposure and their interaction on host survival (using only parasite-exposed animals). This model was significant ( $\chi^2 = 102.53$ , d.f. = 3,  $P < 0.0001$ ;  $n = 167$ ) and included a significant interaction term, reflecting changes in tolerance with host stage (stage  $\times$  infection  $\chi^2 = 13.94$ ,  $P = 0.0002$ ). Re-running the analysis separately for each developmental stage, we found that infection negatively predicted survival only for stages 23–24 ( $\chi^2 = 32.94$ ,  $P < 0.0001$ ;  $n = 54$ ), stage 26 ( $\chi^2 = 22.08$ ,  $P < 0.0001$ ;  $n = 54$ ) and stage 28 ( $\chi^2 = 8.10$ ,  $P = 0.0044$ ;  $n = 27$ ). For later stages (30–37), infection did not significantly predict survival ( $P > 0.05$ ).



**Fig. 3.** The percentage recovery of *Ribeiroia* metacercariae in amphibians examined within 10 days of parasite exposure. Stage of exposure indicates the developmental stage (Gosner 1960) during which the amphibians were exposed to infection. Included are animals that died or were euthanized following exposure. Error bars represent +1 SE.

## Discussion

For more than a century, the ‘gold standard’ for understanding the aetiology of disease has been Koch’s Postulates, which state that the exposure of a healthy host to a disease-causing agent should reproduce the disease if the correct cause has been identified (e.g. Evans 1978). However, and as later recognized by Koch, fulfilment of this particular postulate can often fail, owing to variation in prior host exposure history, asymptomatic carriers and genetic immunity (Evans 1978; Fredericks & Relman 1996; Plowright *et al.* 2008). Host age and developmental stage can also influence both the likelihood a host becomes infected following exposure (resistance) as well as its level of pathology following infection (tolerance). Results of the current study support the hypothesis that pathology in amphibians associated with trematode infection occurs during a ‘critical window’ of development. For a fixed dosage of *Ribeiroia* cercariae, the risk of host death and deformity was greatest among tadpoles exposed during the pre-limb and early limb developmental stages, with steady declines at later developmental stages. The difference of only a few developmental stages – the equivalent of <2 weeks of development under normal conditions – decreased mortality and malformations by >50% (see also Holland *et al.* 2007). Beyond Gosner (1960); stage 30, a low level of parasite exposure had no effect on survival and only weak effects on malformation risk. Parasite exposure also had no effect on amphibian eggs or post-metamorphic frogs, perhaps suggesting that the settlement cues used by cercariae are most pronounced for tadpoles.

These findings support and extend the results of previous work in related systems. Schotthoefer *et al.* (2003b) found

that exposure of *Lithobates pipiens* larvae to variable numbers of *Ribeiroia* cercariae (0–90) produced stage-dependent mortality and malformations, similar to the current study. In a short-term study comparing the effects of different trematode species on larvae of three amphibian species, Rohr, Raffel & Hall (2010) found that developmental stage helped explain changes in both resistance and tolerance among treatments, particularly for *Ribeiroia* exposures. In a pair of related papers, Schotthoefer, Cole & Beasley (2003a) working with *L. pipiens* and Holland *et al.* (2007) working with *Lithobates clamitans* reported that cercariae of the kidney-infecting trematode *Echinostoma* spp. also caused stage-dependent mortality as a function of kidney development, although these effects manifested only at the earliest exposure stages (Gosner stage 25). Sublethal forms of pathology such as whole-body oedema exhibited a similar form of stage dependency (Schotthoefer, Cole & Beasley 2003a; Holland *et al.* 2007).

Nor are such findings restricted to amphibian infections (Cunningham *et al.* 2005; Villeneuve *et al.* 2005). Kelly *et al.* (2009), for instance, reported that experimental exposures with trematode cercariae (*Telogaster opisthorchis*) caused high frequencies (20–65%) of spinal deformities and fin anomalies in juvenile fish (*Galaxias anomalus*) in New Zealand. Based on progressive increases in infection concurrent with a decline in malformations over the season, the authors argued that these abnormalities develop during a critical developmental window of skeletal ossification in the host and that parasite-induced malformations can have population-level impacts on hosts (Kelly *et al.* 2009). Similarly, the myxosporean parasite *Myxobolus cerebralis*, which causes whirling disease in salmonid fishes, is particularly damaging to spinal development in young fishes (Sollid *et al.* 2003). This trend is explained by changes in the availability of cartilage with ossification and in non-specific immunity with fish age (El-Matbouli, Fischer-Scherl & Hoffman 1992; Ryce *et al.* 2005). These observations indicate that critical windows of developmental sensitivity to disease may be common among wildlife infections.

Our study provides additional insights about the relative contributions of host resistance and tolerance driving such developmental variation in disease patterns. While resistance and tolerance are gaining increased attention in animal disease research as a conceptual framework to understand the ecology and evolution of host–parasite interactions (Boots 2008; Read, Graham & Råberg 2008), as of yet there have been few attempts to apply these principles toward the study of wildlife disease or to how disease risk varies with host development (Rohr, Raffel & Hall 2010). Our results indicate that both resistance and tolerance increased with amphibian development. Infection success decreased with developmental stage of exposure even after controlling for time post-exposure, which is an important albeit often neglected determinant of parasite persistence (Johnson & Buller in press). However, this pattern was driven largely by the low infection success in post-metamorphic frogs, for which fewer than 7% of parasites were recovered upon necropsy (see also Schotthoefer, Cole & Beasley 2003a). Exclusion of this group eliminated the

significant effect between stage and infection success. Thus, resistance changed in a strongly non-linear fashion with host development; while larvae were equally susceptible, post-metamorphic frogs (and amphibian eggs) exhibited very high levels of resistance.

These results suggest that changes in host tolerance (rather than resistance) played the most important role in explaining shifts in pathology with host developmental stage. Stage of exposure and infection intensity interacted significantly to explain host mortality, such that infection was a significant predictor of mortality only among early stage tadpoles (Råberg, Graham & Read 2009). For example, while nearly identical levels of infection were recovered among tadpoles exposed to *Ribeiroia* cercariae at stages 34 and 26 (4.1 vs. 4.14, respectively), the groups differed in mortality by 56% (100% survival at stage 34 but only 44% at stage 26). Similarly, both mortality and malformation risk decreased significantly with increases in the developmental stage during which hosts were exposed, regardless of whether post-metamorphic frogs were included in the analysis. The simplest explanation for observed increases in tolerance with host stage involves changes in body size. Older tadpoles at more advanced stages were also considerably larger, which likely reduced the proportional impact of each parasite. Snout-vent length and host stage were strongly correlated ( $r = 0.94$ ), with tadpoles increasing in SVL by >250% between stages 24 and 37 ( $R^2 = 0.89$ ,  $F_{1,86} = 690.3$ ,  $P < 0.0001$ ). Like many trematodes, *Ribeiroia* cercariae use proteolytic enzymes to burrow into host tissue (Kašný *et al.* 2009), a process that causes wounding and micro-haemorrhaging in the process. In small tadpoles, the cumulative effect of multiple infections, each of which penetrates the animal's body, can lead to serious injury from which recovery is unlikely (Rohr, Raffel & Sessions 2009).

Ongoing debates and discussions surrounding amphibian malformations and their causes have often focused on the types of observed malformations and what they indicate about the cause responsible. For example, some reports have suggested that parasite infection is predominantly associated with extra limb abnormalities whereas other causes, such as aquatic predators, may account for missing limb abnormalities (Ballengee & Sessions 2009; Johnson & Bowerman 2010; Skelly & Benard 2010). However, our results indicated that critical windows of development strongly influence the types of malformations observed following exposure. Exposure to *Ribeiroia* cercariae early in development, prior to initiation of limb development (stage 23–24), caused almost exclusively missing limbs, with no extra limbs or limb elements observed. Later exposures (stage 28–30) resulted in an increased frequency of bony triangles and extra limbs or limb-like projections, which declined in scale and severity as developmental stage progressed further. Non-structural abnormalities such as skin webbings and unarticulated outgrowths (femoral projections) were the only abnormalities observed among animals exposed after stage 32, which corresponds with the completion of limb development. Intriguingly, even tadpoles

exposed after completing limb development (e.g. stage 37) exhibited a low frequency of such abnormalities (Fig. 2).

These findings help to explain why within a single wetland, normal and malformed frogs can exhibit similar levels of *Ribeiroia* infection (Goodman & Johnson in press; Johnson & Hartson 2009). Malformation risk depends on the exact position of invading parasites and the developmental stage of the host during infection. Considering that larval amphibians often develop in multiple cohorts and that, even within a single cohort development rate among individuals varies considerably, the effects of parasite exposure on host malformation risk will be context dependent. Infection late in development, for instance, may lead to little or no developmental abnormalities, as shown here, whereas animals exposed early in the growth process have a much greater risk of malformations and mortality, even in response to identical numbers of parasites. These results are in parallel with studies of human exposures to contaminants during critical developmental windows. During the 1950s and 1960s, for instance, administration of thalidomide to pregnant women led to limb, facial and systemic abnormalities in developing embryos depending on the gestation stages at the time of exposure (Smithells & Newman 1992; Tabin 1998).

In conclusion, our results extend and reinforce the importance of critical windows of development in determining the pathology associated with parasite infection. These findings suggest that population-level impacts of infections will vary substantially as a function of the timing of exposure; in some instances, infections are expected to cause mortality and population declines (see Kelly *et al.* 2009). Such effects will be difficult to detect if the age-dependent nature of pathology is not recognized. While changes in both host resistance and host tolerance contribute to this pattern, increases in tolerance with host age/size are particularly important in controlling malformation and mortality risk in larval amphibians. Intriguingly, this suggests that hosts can achieve a size or stage refugium from infection akin to size-based refugia observed in predator–prey dynamics (e.g. Chase 1999); however, this refuge is the result not of a reduction in infection but of a decrease in the resultant damage associated with infection. A pressing arena for future research involves evaluating the effects of climate change on infections with critical windows of pathology. Given that parasites are often more sensitive to changes in temperature than their vertebrate hosts, for instance, anticipated warming could shift infections earlier in the season when hosts are most vulnerable to pathology (see Yang & Rudolf 2009; Gilman *et al.* 2010; Paull and Johnson in press a,b).

## Acknowledgements

We are indebted to E. Daly, M. Redmond, S. Todd, A. Price and M. McGrath for assistance with experimental maintenance and animal husbandry. We further acknowledge J. Hoverman, S. Paull, K. Dosch, S. Orlofske, J. Mihaljevic and M. Joseph for thoughtful suggestions on improving the manuscript. This project was supported by a grant from NSF (DEB-0553768) and a fellowship from the David and Lucile Packard Foundation.

## References

- Astley, S.J., Magnuson, S.I., Omnell, L.M. & Clarren, S.K. (1999) Fetal alcohol syndrome: changes in craniofacial form with age, cognition, and timing of ethanol exposure in the macaque. *Teratology*, **59**, 163–172.
- Baird, J.K. (1998) Age-dependent characteristics of protection v. susceptibility to *Plasmodium falciparum*. *Annals of Tropical Medicine and Parasitology*, **92**, 367–390.
- Ballengue, B. & Sessions, S.K. (2009) Explanation for missing limbs in deformed amphibians. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, **312**, 770–779.
- Beaver, P.C. (1939) The morphology and life history of *Psilostomum ondatrae* Price 1931 (Trematoda: Psilostomidae). *Journal of Parasitology*, **25**, 383–393.
- Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H. & White, J.S.S. (2008) Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology and Evolution*, **24**, 119–174.
- Boots, M. (2008) Fight or learn to live with the consequences? *Trends in Ecology and Evolution*, **23**, 248–250.
- Bowerman, J. & Johnson, P.T.J. (2003) Timing of trematode-related malformations in Oregon spotted frogs and Pacific treefrogs. *Northwestern Naturalist*, **84**, 142–145.
- Bunn, T.L., Parsons, P.J., Kao, E. & Dietert, R.R. (2001) Exposure to lead during critical windows of embryonic development: differential immunotoxic outcome based on stage of exposure and gender. *Toxicological Sciences*, **64**, 57–66.
- Chase, J.M. (1999) Food web effects of prey size-refugia: variable interactions and alternative stable equilibria. *The American Naturalist*, **154**, 559–570.
- Cunningham, M.E., Markle, D.F., Watral, V., Kent, M.L. & Curtis, L.R. (2005) Patterns of fish deformities in the Willamette River, Oregon, and their association with trematode cysts. *Environmental Biology of Fishes*, **73**, 9–19.
- Dally, A. (1997) The rise and fall of pink disease. *Social History of Medicine*, **10**, 291–304.
- El-Matbouli, M., Fischer-Scherl, T. & Hoffman, R.W. (1992) Present knowledge of the life cycle, taxonomy, pathology, and therapy of some *Myxospora* spp. important for freshwater fish. *Annual Reviews of Fish Diseases*, **3**, 367–402.
- Evans, A.S. (1978) Causation and disease: a chronological journey. *American Journal of Epidemiology*, **108**, 249–258.
- Fredericks, D.N. & Relman, D.A. (1996) Sequences-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clinical Microbiology Reviews*, **9**, 18–33.
- Gilman, S.E., Urban, M.C., Tewksbury, J., Gilchrist, G.W. & Holt, R.D. (2010) A framework for community interactions under climate change. *Trends in Ecology and Evolution*, **25**, 325–331.
- Goodman, B.A. & Johnson, P.T.J. (in press) Ecomorphology and disease: understanding the cryptic effects of parasitism on host habitat use, thermoregulation, and predator avoidance. *Ecology*.
- Goodwin, K., Viboud, C. & Simonsen, L. (2006) Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine*, **24**, 1159–1169.
- Gosner, K.L. (1960) A simple table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, **16**, 183–190.
- Heinze, G. & Schemper, M. (2002) A solution to the problem of separation in logistic regression. *Statistics in Medicine*, **21**, 2409–2419.
- Holland, M.P. (2009) Echinostome metacercariae cyst elimination in *Rana clamitans* (green frog) tadpoles is age-dependent. *Journal of Parasitology*, **95**, 281–285.
- Holland, M.P., Skelly, D.K., Kashgarian, M., Bolden, S.R., Harrison, L.M. & Cappello, M. (2007) Echinostome infection in green frogs (*Rana clamitans*) is stage and age dependent. *Journal of Zoology*, **271**, 455–462.
- Johnson, P.T.J. & Bowerman, J. (2010) Do predators cause frog deformities? The need for an eco-epidemiological approach. *Journal of Experimental Zoology*, **314B**, S15–S18.
- Johnson, P.T.J. & Buller, I.D. (in press) Parasite competition hidden by correlation coinfections: using field studies and experiments to understand parasite interactions. *Ecology*.
- Johnson, P.T.J. & Hartson, R.B. (2009) All hosts are not equal: explaining differential patterns of malformations in an amphibian community. *Journal of Animal Ecology*, **78**, 191–201.
- Johnson, P.T.J. & McKenzie, V.J. (2008) Effects of environmental change on helminth infections in amphibians: exploring the emergence of *Ribeiroia* and *Echinostoma* infections in North America. *The Biology of Echinostomes, From the Molecule to the Community* (eds B. Fried & R. Toledo), Chapter 11, pp. 249–280. Springer, New York, NY, USA.
- Johnson, P.T.J. & Thieltges, D.W. (2010) Diversity, decoys and the dilution effect: how ecological communities affect disease risk. *Journal of Experimental Biology*, **213**, 961–970.
- Johnson, P.T.J., Lunde, K.B., Ritchie, E.G. & Launer, A.E. (1999) The effect of trematode infection on amphibian limb development and survivorship. *Science*, **284**, 802–804.
- Johnson, P.T.J., Lunde, K.B., Haight, R.W., Bowerman, J. & Blaustein, A.R. (2001) *Ribeiroia ondatrae* (Trematoda: Digenea) infection induces severe limb malformations in western toads (*Bufo boreas*). *Canadian Journal of Zoology*, **79**, 370–379.
- Johnson, P.T.J., Lunde, K.B., Thurman, E.M., Ritchie, E.G., Wray, S.W., Sutherland, D.R., Kapfer, J.M., Frest, T.J., Bowerman, J. & Blaustein, A.R. (2002) Parasite (*Ribeiroia ondatrae*) infection linked to amphibian malformations in the western United States. *Ecological Monographs*, **72**, 151–168.
- Johnson, P.T.J., Sutherland, D.R., Kinsella, J.M. & Lunde, K.B. (2004) Review of the trematode genus *Ribeiroia* (Psilostomidae): ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. *Advances in Parasitology*, **57**, 191–253.
- Johnson, P.T.J., Reeves, M.K., Krest, S.K. & Pinkney, A.E. (2010) A decade of deformities: advances in our understanding of amphibian malformations and their implications. *Ecotoxicology of Amphibians and Reptiles*, 2nd edn (eds D.W. Sparling, G. Linder, C.A. Bishop & S.K. Krest), pp. 511–536. SE-TAC Press, Pensacola FL.
- Kalter, H. (2003) Teratology in the 20th century: environmental causes of congenital malformations in humans and how they were established. *Neurotoxicology and Teratology*, **25**, 131–282.
- Kašný, M., Mikeš, L., Hampl, V., Dvořák, J., Caffrey, C.R., Dalton, J.P. & Horák, P. (2009) Peptidases of trematodes. *Advances in Parasitology*, **69**, 205–297.
- Kelly, D.W., Thomas, H., Thieltges, D.W., Poulin, R. & Tompkins, D.M. (2009) Trematode infection causes malformations and population effects in a declining New Zealand fish. *Journal of Animal Ecology*, **79**, 445–452.
- Kiesecker, J.M. (2002) Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 9900–9904.
- Kover, P.X. & Schaal, B.A. (2002) Genetic variation for disease resistance and tolerance among *Arabidopsis thaliana* accessions. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 11270–11274.
- Lloyd-Smith, J.O., Schreiber, S.J., Kopp, P.E. & Getz, W.M. (2005) Super-spreading and the effect of individual variation on disease emergence. *Nature*, **438**, 355–359.
- MacLachlan, N.J., Conley, A.J. & Kennedy, P.C. (2000) Bluetongue and equine viral arteritis viruses as models of virus-induced fetal injury and abortion. *Animal Reproduction Science*, **60**, 643–651.
- Mittendorf, R. (1995) Teratogen update: carcinogenesis and teratogenesis associated with exposure to diethylstilbestrol (DES) *in utero*. *Teratology*, **51**, 435–445.
- Paull, S.H. & Johnson, P.T.J. (in press a) Predicting the synergies between climate change and infectious disease. *Conserving Wildlife Populations in a Changing Climate* (eds J. Brodie, E. Post & D. Doak), University of Chicago Press, Chicago.
- Paull, S. & Johnson, P.T.J. (in press b) How will climate change affect host–parasite interactions? Understanding differential responses of hosts and parasites. *Freshwater Biology*.
- Plowright, R.K., Sokolow, S.H., Gorman, M.E., Daszak, P. & Foley, J.E. (2008) Causal inference in disease ecology: investigating ecological drivers of disease emergence. *Frontiers in Ecology and the Environment*, **6**, 420–429.
- Pryor, J.L., Hughes, C., Foster, W., Hales, B.F. & Robaire, B. (2000) Critical windows of exposure for children's health: the reproductive system in animals and humans. *Environmental Health Perspectives*, **108**, 491–503.
- Råberg, L., Graham, A.L. & Read, A.F. (2009) Decomposing health: tolerance and resistance to parasites in animals. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **364**, 37–49.
- Read, A.F., Graham, A.L. & Råberg, L. (2008) Animal defenses against infectious agents: is damage control more important than pathogen control? *PLoS Biology*, **6**, e1000004. DOI: 10.1371/journal.pbio.1000004.
- Rohr, J.R., Raffel, T.R. & Hall, C.A. (2010) Developmental variation in resistance and tolerance in a multi-host–parasite system. *Functional Ecology*, **24**, 1110–1121.
- Rohr, J.R., Raffel, T.R. & Sessions, S.K. (2009) Digenetic trematodes and their relationship to amphibian declines and deformities. *Amphibian Biology Vol.*

8. *Amphibian Decline: Disease, Parasites, Maladies and Pollution* (ed Heatwole H.), Chapter 4, pp. 3067–3088. Surrey Beatty Pty Ltd., Baulkham Hills, NSW, Australia.
- Ryce, E.K.N., Zale, A.V., MacConnell, E. & Nelson, M. (2005) Effects of fish age versus size on the development of whirling disease in rainbow trout. *Diseases of Aquatic Organisms*, **63**, 69–76.
- Schotthoefer, A.M., Cole, R.A. & Beasley, V.R. (2003a) Relationship of tadpole stage to location of echinostoma cercariae encystment and the consequences for tadpole survival. *Journal of Parasitology*, **89**, b475–b482.
- Schotthoefer, A.M., Koehler, A.V., Meteyer, C.U. & Cole, R.A. (2003b) Influence of *Ribeiroia* infection on limb development and survival of northern leopard frogs: effects of host stage and parasite exposure level. *Canadian Journal of Zoology*, **81**, 1144–1153.
- Sessions, S.K. & Ruth, S.B. (1990) Explanation for naturally occurring supernumerary limbs in amphibians. *Journal of Experimental Zoology*, **254**, 38–47.
- Skelly, D.K. & Benard, M.F. (2010) Mystery unsolved: missing limbs in deformed amphibians. *Journal of Experimental Zoology*, **314 B**, 179–181.
- Skerratt, L.F., Garner, T.W.J. & Hyatt, A.D. (2010) Determining causality and controlling disease is based on collaborative research involving multidisciplinary approaches. *EcoHealth*, **6**, 331–334.
- Smithells, R.W. & Newman, C.G.H. (1992) Recognition of thalidomide defects. *Journal of Medical Genetics*, **29**, 716–723.
- Sollid, S.A., Lorz, H.V., Stevens, D.G. & Bartholomew, J.L. (2003) Age-dependent susceptibility of Chinook salmon to *Myxobolus cerebralis* and effects of sustained parasite challenges. *Journal of Aquatic Animal Health*, **15**, 136–146.
- Stopper, G.F., Hecker, L., Franssen, R.A. & Sessions, S.K. (2002) How trematodes cause limb deformities in amphibians. *Journal of Experimental Zoology*, **294**, 252–263.
- Tabin, C.J. (1998) A developmental model for thalidomide defects. *Nature*, **396**, 322–323.
- Tchounwou, P.B., Ayensu, W.K., Ninashvili, N. & Sutton, D. (2003) Environmental exposure to mercury and its toxicopathologic implications for public health. *Environmental Toxicology*, **18**, 149–175.
- Telfer, S., Birtles, R., Bennett, M., Lambin, X., Paterson, S. & Begon, M. (2008) Parasite interactions in natural populations: insights from longitudinal data. *Parasitology*, **135**, 767–781.
- Thompson, W.W., Shay, D.K., Weintraub, E., Brammer, L., Bridges, C.B., Cox, N. & Fukuda, K. (2004) Influenza-associated hospitalizations in the United States. *The Journal of the American Medical Association*, **292**, 1333–1340.
- Villeneuve, D.L., Curtis, L.R., Jenkins, J.J., Warner, K.E. & Tilton, F. (2005) Environmental stresses and skeletal deformities in fish from the Willamette River, Oregon. *Environmental Science and Technology*, **39**, 3495–3506.
- Yang, L.H. & Rudolf, V.H.W. (2009) Phenology, ontogeny and the effects of climate change on the timing of species interactions. *Ecology Letters*, **13**, 1–10.
- Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A. & Smith, G.M. (2009) *Mixed Effects Models and Extensions in Ecology with R*. Springer, New York, NY, USA.

Received 18 October 2010; accepted 16 December 2010

Handling Editor: Jonathan Blount