Negative effects of changing temperature on amphibian immunity under field conditions

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Summary

1. Recent evidence of the important role of emerging diseases in amphibian population declines makes it increasingly important to understand how environmental changes affect amphibian immune systems.

2. Temperature-dependent immunity may be particularly important to amphibian disease dynamics, especially in temperate regions. Changes in temperature are expected to cause deviations away from optimal levels of immunity until the immune system can respond.

3. To test whether temperature changes cause deviations from optimal immunity under natural conditions, we conducted a seasonal survey of adult Red-Spotted Newts and measured basal levels of several immunological variables.

4. We then examined these findings in relation to: (1) the lag hypothesis, which predicts that changes in temperature-dependent immune parameters lag behind short-term temperature changes, and (2) the seasonal acclimation hypothesis, which predicts that immune cell production declines during long-term temperature decreases until amphibians can fully acclimate to winter conditions.

5. Our results supported both hypotheses, showing a spring lag effect on lymphocyte levels and an even stronger seasonal acclimation effect on lymphocytes, neutrophils and eosinophils in the autumn. Our findings suggest that temperature variability causes increased susceptibility of amphibians to infection, and they have implications for the emergence of disease and the potential for climate change to exacerbate amphibian decline.

Key-words: Amphibian decline, climate change, immune, newt, *Notophthalmus viridescens*

Introduction

One of the major questions in ecology is how environmental factors influence the dynamics of parasitism and disease in natural populations. A case in point is that environmental factors have been implicated in the emergence of new and more severe amphibian diseases (Kiesecker 2002; Blaustein *et al*. 2003), which in turn may be contributing to worldwide declines of amphibian populations (Daszak *et al*. 1999; Daszak, Cunningham & Hyatt 2003; Kiesecker *et al*. 2004; Lips *et al*. 2006). In particular, changing climatic conditions have been suggested to be important for amphibian declines in both tropical and temperate regions (Pounds, Fogden

& Campbell 1999; Alexander & Eischeid 2001; Kiesecker, Blaustein & Belden 2001; Rohr & Madison 2003; Daszak *et al*. 2005), and increased infection risk due to warming trends has recently been implicated in the extinction of many tropical frog species (Pounds *et al*. 2006). Environmental temperature has strong effects on the amphibian immune system and may be an important factor influencing susceptibility of amphibians to emerging pathogens (Maniero & Carey 1997; Carey, Cohen & Rollins-Smith 1999; Rojas *et al*. 2005). Effects of temperature on susceptibility have been implicated in outbreaks of chytrid fungus infection, which causes higher mortality at lower temperatures (Woodhams, Alford & Marantelli 2003; Berger *et al*. 2004).

Some components of the amphibian immune system are reduced during periods of low temperature (Maniero & Carey 1997), which may be an adaptive response to

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decreased infection risk during the winter. Host susceptibility is a function of both the strength of the immune response and the intrinsic growth rate of the parasite, an effect often ignored in mammals whose bodies remain at a constant temperature throughout the year (but see Prendergast *et al.* 2002). Unlike mammals, amphibians undergo major seasonal changes in body temperature, which should cause predictably slower pathogen growth rates within the body when the temperature is low (Ratkowsky *et al.* 1982). Even a reduced immune system may be sufficient to deal with pathogens in the seasonally cold environments experienced by temperate amphibians (Schmid 1982; Plytycz & Bigaj 1983; Wojtowicz & Plytycz 1997). Since the immune system is costly to maintain (Bonneaud *et al.* 2003; Ksiazek *et al.* 2003), temperature-dependent immunity could be an adaptive mechanism for ectotherms to save energy during the winter. Results from experimental studies suggest that cold-acclimated fish and amphibians up-regulate immune cell and protein production rates during the winter to counteract the direct effects of temperature on metabolic rate, implying that amphibians could maintain these immune parameters at higher levels in winter if that was adaptive (Bly & Clem 1991; Plytycz & Jozkowicz 1994). We therefore assume that amphibians regulate immune parameters to different levels at different temperatures to optimize fitness, owing to a trade-off between the cost of immunity and temperature-dependent growth rates of amphibian parasites (we will refer to this as the optimal level of immunity for a given temperature).

However, not all amphibian immune parameters respond to temperature in the same way. Lymphocytes, eosinophils and complement activity remain at low basal levels at low temperatures even in winter-acclimated amphibians (held at 4 °C for at least 3 weeks), despite the ability of ectotherms to up-regulate lymphocyte production in winter. This observation suggests these immune parameters have temperature-dependent optimal levels (Green & Cohen 1977; Bly & Clem 1991; Maniero & Carey 1997), so we will refer to these as 'temperature-dependent' immune parameters. Neutrophils and phagocytic activity initially decrease when temperature drops but are brought back to high levels once amphibians acclimate to the lower temperature, suggesting temperature-independent optima for these immune parameters (Plytycz & Jozkowicz 1994; Maniero & Carey 1997).

When temperatures vary, maintaining optimal immune status may not be possible. Based on the results of laboratory studies, we have formulated two hypotheses for how temperature changes should influence the amphibian immune system, which we will call the 'lag effect' and the 'seasonal acclimation effect'. The lag effect is a hypothesized delay in the adjustment of immune parameters to their new optimal levels following a rapid temperature change, owing to the length of time it takes to produce or remove a given immune cell or protein. During periods of increasing temperature, the length of this delay should be limited by the length of time necessary to produce a given immune cell or protein (e.g. 7–11 days for development of stem cells into mature leucocytes; Bell & Hughes 1997). In support of this hypothesis, Maniero & Carey (1997) found that it took 7–9 days for complement activity in frogs to increase to its new level following an abrupt temperature increase. During periods of decreasing temperature, this delay should be determined by the rate of removal of immune cells or proteins from the blood (e.g. half-life of 3–8 h for eosinophils, basophils and neutrophils and 3–8 weeks for lymphocytes; Bell & Hughes 1997; DeSantis & Strauss 1997; Janeway *et al.* 2001).

The seasonal acclimation effect is a hypothesized change in the rate of cell or protein production (i.e. number of cells or proteins produced per day) above or below optimal rates immediately following seasonal temperature increases or decreases, respectively, due to slow acclimation of amphibians to seasonal temperature extremes. Note that even if each of these cells still takes 7–11 days to complete development as with the lag effect, the rate of production will be higher if more cells are going through that process at any given moment. Cold-acclimated fish and amphibians maintain higher levels of phagocytic activity, antibody production and lymphocyte numbers at cold temperatures than warm-acclimated control animals recently moved to cold temperatures (Bly & Clem 1991; Plytycz & Jozkowicz 1994). Conversely, cold-acclimated fish and amphibians moved to warm temperatures produce similar or slightly elevated levels of macrophage activity compared with warm-acclimated control animals (Plytycz & Jozkowicz 1994). These results suggest that ectotherms adjust metabolic pathways during the winter to accelerate the production of immune cells and proteins, particularly at low temperatures. Bly & Clem (1991) found that it takes 4–6 weeks for fish lymphocytes and antibody activity to return to stable levels following a rapid drop in temperature, suggesting this type of acclimation probably occurs only during long-term seasonal changes in temperature.

Although each of these effects involves a time delay and a type of acclimation, they are caused by different mechanisms acting on different time-scales and predict different responses to temperature changes. The lag hypothesis predicts lower than optimal levels of temperature-dependent immune parameters following short-term (8–14 days) temperature increases and higher than optimal levels following short-term (1–2 days for eosinophils and neutrophils) temperature decreases (Fig. 1a). The seasonal acclimation hypothesis predicts lower than optimal levels of immune parameters relative to temperature following long-term (30–60 days) seasonal temperature decreases and slightly elevated levels following long-term temperature increases (Fig. 1b). The latter hypothesis applies to any immune cell or protein whose production rates are influenced by seasonal acclimation, including and perhaps especially those that are maintained at high levels during the winter (i.e. temperature-independent by our definition).
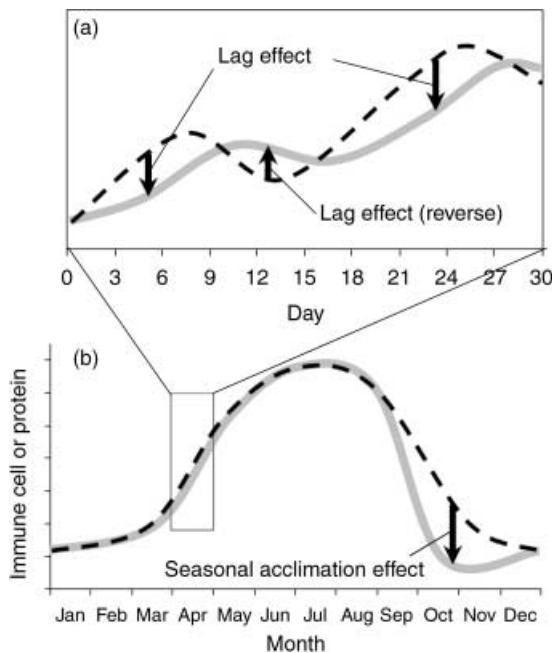
Fig. 1. Hypothesized effects of changing temperature on amphibian immunity. The dotted curves indicate optimal levels of a hypothetical temperature-dependent immune parameter, and the grey curves indicate the actual levels of this immune parameter predicted by the lag and acclimation effect hypotheses at different time-scales. Temperature is assumed to exhibit both within and between-season fluctuations.

These hypotheses are not mutually exclusive, and both may influence levels of immune parameters following temperature changes. The fine-scale adjustments to short-term temperature changes relevant to the lag effect might be considered analogous to changing speeds within gears in an automobile, whereas seasonal acclimation would be analogous to shifting gears, since different metabolic processes appear to be at work in the winter from those in the summer.

Despite numerous laboratory studies of fish and amphibian immunity, the lack of published field data makes it difficult to assess the importance of temperature to amphibian immunity under complex natural conditions, such as seasonal cues which might allow amphibians to anticipate temperature changes (Delgado, Alonsogomez & Alonsobedate 1992). The goal of this study was to track seasonal changes in the immune system of free-living adult amphibians in order to address the following questions: (1) how do patterns of temperature-dependent immunity in wild amphibians compare with laboratory results, (2) do amphibians experience seasonal variation in immunity above and below temperature-dependent optima, and (3) is this variation consistent with the lag and seasonal acclimation hypotheses? We chose the Red-Spotted Newt (*Notophothalmus viridescens*) as a model organism because adult newts are active in ponds throughout the year (Petranka 1998), allowing sampling from the same habitat in all seasons, and have a variety of responses that are strongly temperature- and season-dependent (Rohr,

Madison & Sullivan 2002; Rohr, Madison & Sullivan 2003). We used basal levels of peripheral neutrophils, eosinophils, basophils and lymphocytes, as well as stomach lysozyme activity, as measures of immune status.

Methods

SURVEY

Five ponds known to support newt populations were chosen in and around Centre County to represent a variety of adult newt habitats. Mothersbaugh (40°39′12″ N, 77°54′9″ W) is a flat-bottom beaver pond in the Penn State Experimental Forest which has decreased considerably in size and depth since the beavers left several years ago. Turtle Shell Pond (40°52′26″ N, 78°4′36″ W) is one of several beaver ponds along a stream system in Moshannon State Forest. Mystery Newt Pond (40°45′53″ N, 78°0′49″ W) and Twin Pond (40°46′49″ N, 78°0′14″ W) are semipermanent (drying some winters) landlocked woodland ponds in the Scotia Barrens area (PA State Game Lands #176), and Little Acre (40°48′6″ N, 77°56′37″ W) is a landlocked permanent pond also located in the Scotia Barrens. Seasonal surveys were conducted in 2003 and 2004 in the spring (March–April), summer (July–August), autumn (September–November) and winter (January–February). A late spring (May–June) survey was added in 2004 and replicated in 2005 following observations of major differences in newt ecology between early and late spring.

NEWT COLLECTION

At each sampling time point, dip nets were used to collect approximately 10 newts per pond for blood collection and dissection (402 total newts). During the winter survey, newts were collected by drilling holes in the ice and setting minnow traps on the bottom of the pond overnight. Newts could not be obtained from Twin Pond in the autumn and winter or from Mystery Newt Pond during the winter. Similar numbers of male and female newts were collected when possible, leading to approximately constant male : female sex ratios across seasons except in winter (early spring 67:33, late spring 58:46, summer 53:35, autumn 45:26, winter 33:6). The strong male bias observed in winter may have been due to a higher trapping success of males, which have higher activity levels than females (Rohr *et al.* 2003). These male : female ratios reflect natural male biased sex ratios in newts (Harris, Alford & Wilbur 1988; Rohr *et al.* 2002, 2003). For each pond at each time point, we recorded water temperature at a depth of 10 cm below the water surface using a YSI Model 95 meter (YSI Incorporated, Yellow Springs, OH). Newts were transported to the lab in 250 ml Nalgene containers filled with pond water, anesthetized with a drop of Oragel® on the head, and euthanized by decapitation within 3 h of collection to minimize effects of transportation stress on the newt immune system.

LEUCOCYTE COUNTS

Several immune system parameters were obtained from blood cell counts. Neutrophils are important phagocytic cells that rapidly respond to infections by a wide variety of parasites, and eosinophils and basophils help defend against larger parasites, such as parasitic helminths, which cannot be engulfed by phagocytes (Janeway *et al.* 2001). Lymphocytes are the primary cells of the host's adaptive immune response to infection (Janeway *et al.* 2001), and the effects of temperature on peripheral lymphocyte levels parallel effects on other measures of adaptive immunity, such as antibody responses and the size of the peripheral lymphoid tissues (Cooper *et al.* 1992). Blood was collected from each newt immediately after euthanasia with a heparinized capillary tube and 3–5 µl smeared on a glass microscope slide. Slides were air dried for 10 min, fixed in methanol for 5 min, and again allowed to dry. A variation of a benzidine staining procedure was used to aid differentiation of erythrocytes from other blood cell types (Beug *et al.* 1982). Slides were placed in 1% *o*-dianisidine (3,3′-dimethoxybenzidine, Sigma, St. Louis, MO) in methanol for 90 s, destained in 1% hydrogen peroxide in 50% ethanol for 90 s, and rinsed twice in deionized water for 30 s. Slides were then counterstained in Giemsa stain for 30 min and rinsed again in deionized water for 30 min. Blood cells were counted at 400× magnification starting in the upper left corner of the smear and working down the slide by moving the objective 2 mm between fields and counting all cells in each field until the erythrocyte count reached 5000. Leucocytes were identified as lymphocytes, thrombocytes, neutrophils, eosinophils, basophils or monocytes according to descriptions in Schermer (1967), Hadji-Azimi *et al.* (1987) and Ussing & Rosenkilde (1995), and were quantified as cells per 5000 erythrocytes. This method of quantifying leucocytes was possible because of high leucocyte/erythrocyte ratios in salamanders.

STOMACH LYSOZYME ACTIVITY

Lysozyme activity in stomach tissue was assayed as a measurement of innate immunity. Lysozyme breaks down bacterial cell walls and provides a first line of defence against bacteria, particularly in the gut mucosa (Lindsay 1986). Newt stomachs were removed, cut in half to remove the contents, and rinsed briefly in deionized water. An equivalent mass of phosphate-buffered saline (pH = 7·2) was added to each sample before samples were frozen at −20 °C. Stomachs were ground to a homogenate using a pellet pestle (Kontes Glass Co., Vineland, NJ). Lysozyme activity was assayed using a lysoplate assay described by Yousif *et al.* (1991), and 15 µl of sample was put into wells (3·5 mm diameter × 4 mm deep) cut into 0·5% agarose in 100 mm diameter Petri dishes. The agarose contained 0·06 M phosphate buffer (pH 6·5), 0·02 M NaCl and 0·6 mg ml^{-1} freeze-dried *Micrococcus leisodeikticus* (Sigma). The diameters of zones of lysis were measured after 20 h incubation at room temperature and 100% humidity. Activity levels were calculated by comparison with zones of lysis produced by hen egg-white lysozyme (Sigma) at standard concentrations (5, 10, 15, 25, 50, 100, 250, 500 and 1000 µg ml^{-1}), the natural log of which relates linearly to the area of the zone of lysis (data not shown).

RECONSTRUCTION OF POND TEMPERATURE PROFILES

Temperature data were collected from all five ponds every 2 h from 7 March to 1 June in the spring of 2005, using temperature data loggers (HOBO, Onset, Pocasset, MA) set at a depth of 20 cm to ensure they would remain submerged as water levels fluctuated. Hourly air temperature data for State College were obtained from a database maintained by the PA State Climatologist website (Bahrmann & Ayers 2005) and used to calculate average daily temperatures for the past 3 years. Average daily air temperature was correlated with average daily temperatures of each pond, providing that the presence of ice was taken into account ($r = 0·56–0·75$ for all ponds when free of ice; $r = 0·35–0·55$ for iced-over ponds with flowing water; temperature approximately constant for iced-over ponds with no water flow). The relationships between each pond's spring 2005 temperatures and air temperature, in addition to observations of the pond melting times in 2003 and 2004, were used to reconstruct estimated temperature profiles for each of the ponds from 2003 to 2005.

STATISTICAL ANALYSES

Generalized linear models were used for all analyses. All blood count data were found to fit the negative binomial error distribution, and lysozyme activity fitted the gamma distribution. Between-population differences were not a focus of this study, so collection site ('Pond') was included in all models as a blocking variable.

To determine which immune parameters had temperature-dependent optima, the effects of temperature on immune parameters were first analysed using Pond as a blocking variable. Temperature varied greatly by season, so to determine temperature-independent effects of season on immune parameters, temperature was included as a covariate and year and pond were included as blocking variables. Because data from late spring were recorded in 2004 and 2005 while data from all other seasons were recorded in 2003 and 2004, data from the late spring surveys were left out of this analysis to avoid an unbalanced design. To test for temperature-independent differences between individual seasons, residuals were calculated from models including pond and temperature and analysed with multiple comparisons tests. Temperature was excluded from analyses of seasonal effects for immune parameters not found to be temperature-dependent in the first analysis (i.e. basophils and lysozyme).

Table 1. Regression statistics describing generalized linear models for the between-season effects of temperature on immune parameters (blocked by Pond). χ^2 = change in deviance when predictor removed from full model

Immune parameter	Source of variation	Coefficient	df	χ^2	P
Lymphocytes	Pond		4	16·3	0·0027
	Temperature	**0·053**	**1**	**263·1**	**<0·0001**
Eosinophils	Pond		4	51·0	<0·0001
	Temperature	**0·116**	**1**	**241·9**	**<0·0001**
Neutrophils	Pond		4	34·1	<0·0001
	Temperature	**−0·012**	**1**	**6·5**	**0·0107**

*See Fig. 2 for plotted data.

To test the lag and acclimation effect hypotheses, immune parameters with significant main effects of temperature were analysed for evidence of effects of temperature change. Since different effects were expected depending on whether newts were acclimated to winter or summer temperatures, data were divided between warm-acclimated (summer and autumn) and cold-acclimated (winter and spring) newts for this analysis. The average rates of temperature change (°C day^{-1}) over the previous 8, 14, 30 and 60 days were calculated for each sampling date in each pond using estimated average daily temperatures from the reconstructed pond temperature profiles. These time-scales were chosen to reflect the time-scales hypothesized to be important for the lag and acclimation effects (lag effect 1–2 weeks, acclimation effect 1–2 months). To control for the main effect of temperature on immune parameters, residuals were calculated from models including pond and temperature as predictor variables. These residuals, which represent deviations away from temperature-dependent optima, were regressed against rates of temperature change using normal errors. A backward selection procedure was used to determine which time-scale(s) of temperature change were the best predictors of variation in immune parameters of cold-adapted and warm-adapted newts. Since four scales of temperature change were being compared simultaneously, Bonferroni-adjusted P-values were used to exclude model parameters (i.e. $P < 0.0125$ to include in model).

Results

There were strong effects of temperature on circulating lymphocytes and eosinophils (Tables 1 and 2, Fig. 2a,b). Neutrophils had a significant negative between-season relationship with temperature (Table 1, Fig. 2c). Neither basophils ($\chi^2 < 0.01$, df = 1, $P = 0.955$) nor lysozyme activity ($\chi^2 = 0.01$, df = 1, $P = 0.926$) showed significant effects of temperature.

Significant seasonal effects were still detected for lymphocytes, eosinophils, neutrophils and lysozyme activity after the direct effect of temperature had been removed (Table 2, Fig. 2). Basophils showed no significant seasonal effects ($\chi^2 = 7.35$, df = 3, $P = 0.061$). Lymphocytes fell below expected levels in the autumn (i.e. lower than could be accounted for by temperature

Table 2. Regression statistics describing minimal generalized linear models for effects of season and temperature on immune parameters (blocked by Pond). Only lysozyme showed a significant main effect of year, but eosinophils showed a significant year by season interaction. χ^2 = change in deviance for each predictor when removed from the full model

Immune parameter	Source of variation	df	χ^2	P
Lymphocytes	Pond	4	11·2	0·0242
	Temperature	**1**	**5·8**	**0·0157**
	Season*	**3**	**50·0**	**<0·0001**
Eosinophils	Pond	4	27·3	<0·0001
	Year	1	0·7	0·4115
	Temperature	**1**	**6·6**	**0·0104**
	Season*	**3**	**10·0**	**0·0187**
	Year:Season*	3	26·2	<0·0001
Neutrophils	Pond	4	16·6	0·0024
	Temperature	**1**	**5·8**	**0·3505**
	Season*	**3**	**40·9**	**<0·0001**
Lysozyme	Pond	4	7·2	0·0922
	Year	1	6·1	0·0054
	Season*	**3**	**24·6**	**<0·0001**

*See Fig. 3 for plotted data.

alone) and returned to higher-than-expected levels in the winter, a pattern that was consistent between years (Fig. 3a). Lymphocytes were also lower than expected in early spring, especially in 2003, and showed a similar pattern in late spring 2005 (Fig. 3a). Eosinophils had lower-than-expected levels in early spring and autumn of 2003 but showed no apparent seasonal pattern in 2004, leading to a significant year-by-season interaction (Fig. 3a, Table 2). Neutrophils decreased below expected levels in the autumn, especially in 2003, followed by an increase in the winter (Fig. 3c). Lysozyme activity followed a different seasonal pattern, with a strong increase in the middle of summer in both years followed by a gradual decrease during the rest of the year to very low levels in late spring (Fig. 3d).

For analyses examining how temperature change influenced immunity, only the 14-day time-scale was significant for tests of the lag effect and only the 60-day time-scale was significant for tests of the seasonal acclimation effect (Table 3), time-scales that are consistent with the predictions for each hypothesis. Cold-acclimated newts, which were predicted to experience
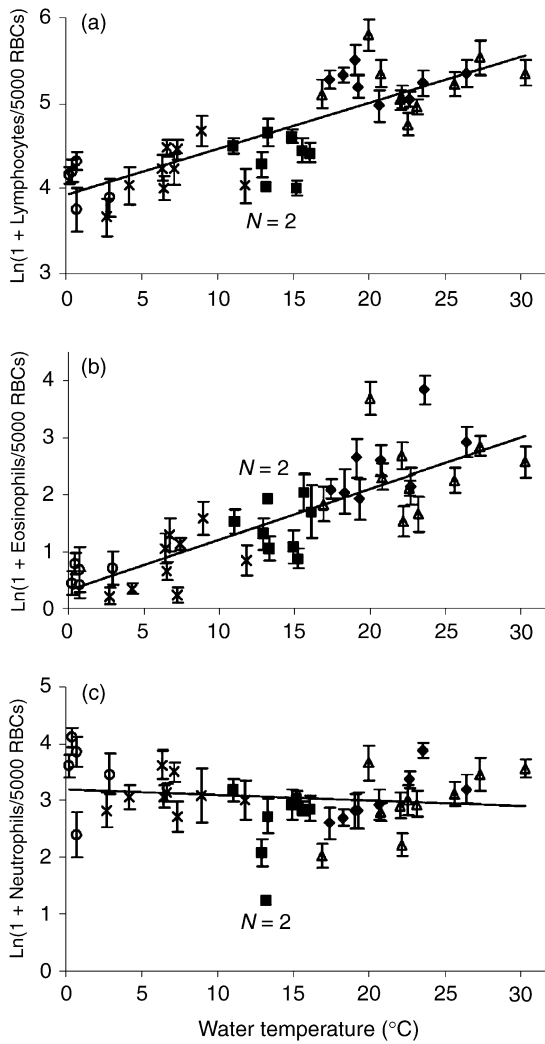
Fig. 2. Between-season effects of temperature on immune parameters (log-transformed). Symbols represent the average and standard error for each pond at each sampling date ($N = 10$ for most points). Different symbols represent different seasons to illustrate seasonal deviations from expected values (✖ early spring, △ late spring, ◆ summer, ■ autumn, and ○ winter).

a lag but not an acclimation effect, showed a significant lag in lymphocyte production in response to short-term temperature changes. Numbers of lymphocytes, but no other immune parameters, were

greater than expected with temperature declines and less than expected with temperature increases (Table 3, Fig. 4a). Warm-acclimated newts, which were predicted to experience an acclimation effect during seasonal temperature decreases and a lag effect during short-term temperature changes, exhibited only a strong acclimation effect for lymphocytes, neutrophils and eosinophils (Table 3, Fig. 4b,c). Newts had significantly lower than expected numbers of these cells if temperatures, on average, had been declining over the past 60 days. This effect accounted for some of the between-year and between-season variability in immune parameters for warm-acclimated newts (Fig. 4b,c), and was larger in magnitude than the lag effect on lymphocytes (as shown by larger coefficients for these models, Table 3).

Discussion

The effects of temperature on immune parameters of wild newts were highly consistent with results from laboratory studies on anuran amphibians. The strong positive between-season temperature dependence of circulating eosinophils and lymphocytes, the weak negative between-season relationship between temperature and neutrophil counts, and the lack of temperature-dependence in basophils were all consistent with the findings of Maniero & Carey (1997) in their laboratory study of Leopard Frog immunity. These similarities suggest that overall effects of temperature on the amphibian immune system are robust to experimental conditions and may be generalized across amphibian taxonomic groups. The cause of the strong temperature-independent seasonal patterns for lysozyme activity remains unresolved.

Circulating lymphocyte levels showed patterns consistent with the lag effect hypothesis in the spring. As predicted, cold-acclimated newts had lower than expected lymphocyte levels following rapid, short-term (14-day) increases in temperature, which helps account for the lower than expected levels observed in early spring 2003 and late spring 2005. Despite low eosinophil levels in early spring 2003, the temperature-change analysis did not provide evidence of a spring lag effect for this immune parameter.

Table 3. Regression statistics describing minimal models for the effects of temperature changes on deviation of immune parameters from their temperature-dependent optimal values (residuals from models described in Table 1). Residuals for cold-acclimated newts (sampled in winter or spring) and warm-acclimated newts (sampled in summer or autumn) were analysed separately. Only a single time-scale of temperature change (8, 14, 30 or 60 days) was a significant predictor for any of the analyses

Immune parameter	Temperature-change time-scale*	Coefficient	df	F	P
Cold-acclimated newts					
Lymphocytes	14 days*	−0·636	1	7·4	0·0070
Warm-acclimated newts					
Lymphocytes	60 days*	5·941	1	38·5	0·0000
Eosinophils	60 days*	5·079	1	20·2	0·0000
Neutrophils	60 days*	3·189	1	11·8	0·0007

*Number of days over which the rate of temperature change was estimated. See Fig. 4 for plotted data.
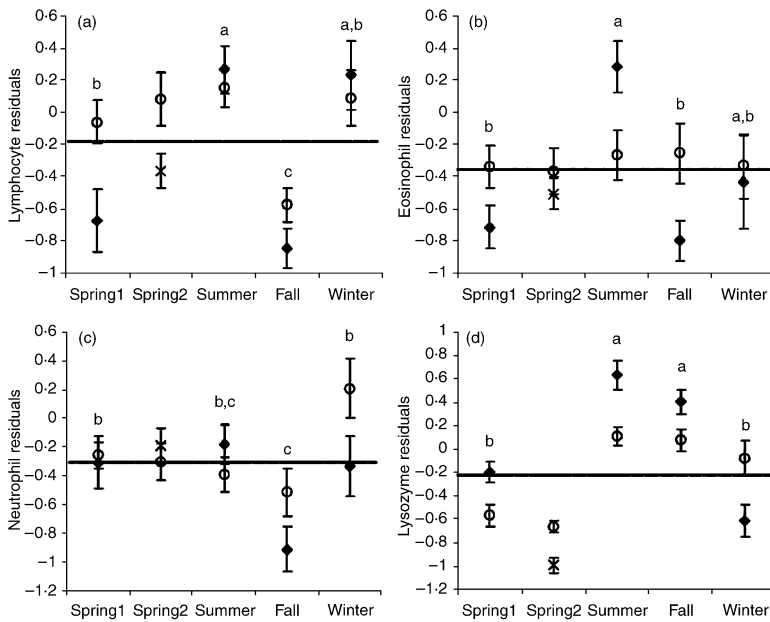
Fig. 3. Seasonal effects on immune parameters, once the effects of temperature had been accounted for. Seasons that were not significantly different from each other ($P > 0.05$) by the multiple comparisons analysis are labelled with the same letter. Patterns differed between years for some immune parameters (◆ 2003 survey, ○ 2004 survey, ✕ 2005 spring survey). Residuals were calculated from generalized linear models including Pond and Temperature (only Pond for lysozyme residuals, see Methods). Error bars represent standard errors, and dashed lines show expected values for immune parameters (residual means, which can be non-zero if error distributions are non-normal).
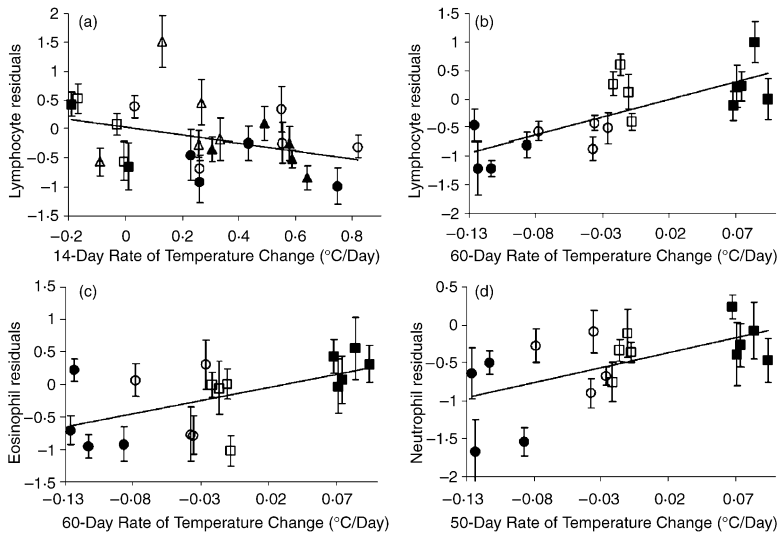
Fig. 4. Effects of changing temperature on immune parameters for cold-acclimated (a) and warm-acclimated (b–d) newts. Different symbols represent different seasons (a winter, ■ early spring, ● late spring; b–d ■ summer, ● autumn). Rates of temperature change helped explain between-season and between-year (open symbols = 2004 survey) differences in residual values of immune parameters. Residuals were calculated from generalized linear models including Pond and Temperature, and symbols represent the average and standard error for each pond at each sampling date ($N = 10$ for most points).

Lymphocytes, eosinophils and neutrophils all showed patterns consistent with predictions of the seasonal acclimation hypothesis. All three immune parameters fell below expected levels in the autumn, except in 2004 when eosinophils remained at expected levels. As pre-

dicted, these decreases could be accounted for by the rate of temperature decrease over the last 60 days, but not for shorter time-scales. The seasonal acclimation effect in autumn appears to be greater in magnitude than the spring lag effect and probably affects newts for a larger proportion of the year owing to the long time-scale over which it operates. This may lead to a period in the autumn during which amphibians predictably experience increased susceptibility to parasites and pathogens.

The absence of a seasonal acclimation effect in spring is unsurprising, given that cold-acclimated fish and amphibians produce similar levels of immune parameters at warm temperatures compared with warm-acclimated control animals (Plytycz & Jozkowicz 1994). The absence of a detectable reverse lag effect in autumn may be due to rapid turnover rates of most immune cells (Bell & Hughes 1997), which should therefore closely match cell production rates as temperature decreases. However, lymphocytes have relatively long half-lives (3–8 weeks, Janeway *et al*. 2001) and might have been expected to show a detectable reverse lag effect in autumn. The apparent dominance of the seasonal acclimation effect in our results may be due to very slow acclimation of newts to winter conditions, as indicated by the long time-scale (60 days) of the effect we observed. Alternately, high levels of parasite antigens in newts may increase the proportion of rapidly cycling lymphocytes owing to increased activation and removal of these cells (Tough & Sprent 1995).

The unfortunate need to use a different sampling technique during winter poses a problem for interpreting our results because of the potential effects of trapping stress. Handling stress causes a substantial decrease in the number of circulating lymphocytes in the hours following mist-net capture of wild birds (Davis 2005), and amphibians have been shown to experience elevated levels of stress hormones following prolonged capture stress (Coddington & Cree 1995). However, increased stress due to trapping is unlikely to have caused the patterns we observed. Acute stress generally leads to short-term decreases in circulating lymphocytes in amphibians (Maule & VanderKooi 1999), opposite the winter effect observed in this study. We know too little about context-dependent stress responses in amphibians to rule out the possibility that newts respond to handling stress differently in different seasons, but this effect is unlikely to have caused the seasonal patterns observed in this study. Amphibians have a slower glucocorticoid response to handling stress than do birds (3–12 h *vs* 5–15 min, Coddington & Cree 1995; Romero & Romero 2002), making immune parameters unlikely to have substantially changed within the 3-h interval between capture and blood collection.

The lower than expected levels of circulating immune cells in the autumn could be attributed to low parasite abundance, breeding, seasonal changes in sex ratios of sampled newts, or stress due to high population density (Zerani & Gobbetti 1993; Rollins-Smith 2001;

Kortet *et al.* 2003), but the seasonal acclimation effect seems like the parsimonious explanation for this pattern. Most parasites of Red-Spotted Newts which have been found to have seasonal patterns infect them through the spring and summer, leading to high prevalence in summer and autumn and low in winter and early spring (Holl 1932; Rankin 1937; Jarroll 1979; Joy & Pennington 1998). Newts would be predicted to have high levels of immune parameters in autumn (similar to those in summer) if seasonal patterns reflected a response to current infection. Similarly, densities of adult newts in ponds peak in the spring, dip to very low levels in the summer and increase again only slightly in the autumn (Gage 1891; Harris *et al.* 1988; T. R. Raffel, personal observation), offering a potential alternative explanation for low immunity in the spring but not in the autumn. Although breeding may influence newt immune parameters, breeding seems unlikely to have caused the observed seasonal patterns of immunity. The newt breeding season starts in the autumn and continues through winter to the following late spring (Gage 1891; Harris 1987; Rohr *et al.* 2002; T. R. Raffel, personal observation), predicting low immunity in the winter as well as in the autumn and spring. Likewise, seasonal differences in the sex ratio of sampled newts cannot explain the observed patterns, since winter was the only season when sex ratio was substantially different. Although a combination of these factors cannot be entirely ruled out, they fail to explain our results as well as the seasonal acclimation hypothesis does. Owing to the presence of confounding variables in our study, experimental studies will be necessary to confirm whether the lag and seasonal acclimation effects are sufficient to explain the patterns we have observed.

The effects of short-term lags in immunity on infection rates have only been tested with a small number of parasites. Maniero & Carey (1997) found that Leopard Frogs took 7–9 days to increase complement activity to expected levels following an abrupt temperature increase, but found no effect of increasing temperature on susceptibility to *Aeromonas* infection. Similarly, Jackson & Tinsley (2002) were unable to detect a lag effect of increasing temperature on amphibian susceptibility to infection by a monogenean parasite.

Effects of cold acclimation to the ectothermic immune system have been best studied in fish. Lymphocytes had much higher peripheral blood counts, proliferation potential and antibody responses at cold temperatures when fish were cold-acclimated, and it took between 4 and 6 weeks for warm-acclimated fish to raise immunity to the same levels as cold-acclimated fish following an abrupt temperature decrease (Bly & Clem 1991). This appears to be an important cause of outbreaks of the fungal disease saprolegniosis on fish farms, which often follow rapid drops in temperature (Bly *et al.* 1993). Cold-acclimation of the immune system has been less extensively studied in amphibians; however, Plytycz & Jozkowicz (1994) found that macrophages from cold-acclimated fish and amphibians had higher activity levels than macrophages from warm-acclimated amphibians when assayed at cold temperatures. Jackson & Tinsley (2002) found that frogs were more susceptible to helminth infection after temperature was lowered than when temperature was held constant or increased, a result consistent with the acclimation effect hypothesis. Further studies are needed to determine the length of time needed for amphibians to acclimate their immune systems to winter conditions, the magnitude of the acclimation effect in the absence of confounding variables, and whether or not other parasites show increased infectivity following temperature decreases.

Our results have implications for how temperature changes might affect disease dynamics in amphibians. Although the decrease in immunity during autumn may not strongly influence infection rates of parasites which peak in the spring and summer, there may be an impact on the ability of newts to clear these parasites, which have often built up to high levels by the end of summer (Holl 1932; Rankin 1937; Jarroll 1979; Joy & Pennington 1998). The lag and acclimation effects may also lead to outbreaks following unusual climatic events, or to the evolution of parasite life-history strategies taking advantage of predictable periods of increased susceptibility. In addition, populations of amphibians having few cold-tolerant resident parasites might invest relatively little energy in immunity during colder seasons, making them more susceptible to invasion by parasites such as chytrid fungus which grow well at low temperatures (Berger *et al.* 2004). Finally, the increased variability in climatic conditions predicted by some climate change scenarios might lead to longer or more frequent periods of immune suppression in amphibians, which could exacerbate amphibian declines (Hegerl *et al.* 2004; Schar *et al.* 2004).

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