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Parasite age-intensity relationships in red-spotted newts: Does immune memory influence salamander disease dynamics? ☆

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ABSTRACT

Acquired immune memory in vertebrates influences transmission and persistence of infections, with consequences for parasite dynamics at both the individual and population levels. The potential impact of acquired immunity is of particular interest for salamanders, whose acquired immune systems are thought to be less effective than those of frogs and other tetrapods. One way to examine the importance of acquired immunity to parasite dynamics at the population level is by examining the relationship between host age and parasite infection intensity. Acquired immunity reduces infection rates in older animals, causing decreased parasite intensity in older age classes and leading to curvilinear age-intensity relationships for persistent parasites and convex age-intensity relationships for transient parasites. We used age-intensity relationships to look for the signature of acquired immunity for 12 parasite taxa of red-spotted newts (*Notophthalmus viridescens*), using data from a 2-year parasitological survey of six newt populations. We estimated ages from snout-vent length (SVL) based on the relationship between SVL and skeletochronologically-derived ages in a subset of newts. We found evidence of acquired immunity to two parasite taxa, bacterial pathogens and the protist *Amphibiocystidium viridescens*, whose convex age-intensity relationships could not be easily explained by alternative mechanisms. Our results suggest that the acquired immune response of newts is sufficient to influence the dynamics of at least some parasites.

1. Introduction

A central question in the study of disease dynamics is: what is the role of acquired immunity (immune memory) at the individual level and how does this response influence parasite dynamics at the population level (Anderson and May, 1985a; Hudson and Dobson, 1995; Wilson et al., 2002)? The success of some vaccination campaigns, in addition to numerous epidemiological and experimental studies, attests to the relevance of acquired immunity in protecting individuals and populations of mammals, birds and even fish against parasitic infections (Brochier et al., 1991; Vaughan et al., 1993; Rohani et al., 2000; Bjornstad et al., 2002; Cattadori et al., 2005; Ge et al., 2007). However, we know little about the relevance of immune memory in natural amphibian populations. Given recent evidence that emerging diseases are responsible for many declines and extinctions of amphibians worldwide (Daszak et al., 1999; Stuart et al., 2004), understanding the importance of acquired immunity in amphibian host-parasite dynamics could improve our ability to understand and respond to worldwide amphibian declines (Carey et al., 1999).

Comparative studies have shown that frogs and salamanders possess the fundamental components of the vertebrate acquired immune system, including the major histocompatibility complex (MHC) class I and II proteins, T-cell receptors and immunoglobulins (Charlemagne and Tournefier, 1998). The speed and diversity of antibody and T-cell responses in the model frog species *Xenopus laevis* appear to be similar to those of birds and mammals (Kaufman and Volk, 1994). Studies of *X. laevis* have shown improved resistance following repeated exposure to helminth and ranavirus infections (Jackson and Tinsley, 2001; Gantress et al., 2003; Maniero et al., 2006). Improved resistance to the latter is clearly mediated by components of the acquired immune system (Robert et al., 2005; Maniero et al., 2006). Recent studies have found increased resistance of some anuran species to chytridiomycosis, a fungal disease, after an initial infection has been eliminated (Cynthia Carey, University of Colorado, personal communication). It therefore seems clear that immune memory is an important and potent part of the frog immune response which might limit parasite transmission and consequently the subsequent dynamics of infection.

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The acquired immune system of salamanders has generally been considered more primitive, or at least slower and less diverse, than the acquired immune systems of frogs and other tetrapods (Kaufman and Volk, 1994). This conclusion stems largely from research with the axolotl (*Ambystoma mexicanum*), a neotenic species used extensively for the study of salamander physiology (Kaufman and Volk, 1994; Charlemagne and Tournefier, 1998). Axolotls lack the MHC receptor diversity characteristic of the acquired immune system in mammals, birds and *X. laevis* and their immune responses appear to take longer to develop. Other salamander species, including adult red-spotted newts (*Notophthalmus viridescens*), also have slow rates of graft rejection, antibody production and mitogen-induced responses compared to anurans and other tetrapods (Kaufman and Volk, 1994). Experimental studies of ranavirus in larval tiger salamanders (*Ambystoma tigrinum*) have so far failed to detect evidence of improved resistance following elimination of an initial infection (Danna Schock, University of Calgary, personal communication). These studies suggest that acquired immunity plays a minor role in salamander immune defense compared with acquired immunity in frogs, although whether these responses are sufficient to influence infection dynamics at the population level remains an open question.

From a phylogenetic perspective it might appear unlikely that salamanders would exhibit a less effective acquired immune response than frogs, which are more closely related to salamanders than they are to birds and mammals (Hedges et al., 1990). This would imply either that the potent acquired immune systems of frogs and mammals evolved independently, which seems unlikely, or that salamanders have lost much of their original immune capacity since their divergence from frogs. Given the ubiquity of parasites in free-living animals (Roberts and Janovy, 2000), the number of shared parasites between frogs and salamanders (Aho, 1990), and similarities in their usage of terrestrial and aquatic habitats (Stebbins and Cohen, 1995), it seems unlikely that salamanders have experienced less intense selection pressure to combat infection than have their anuran counterparts. Furthermore, findings of increased antibody and graft rejection responses following repeated exposure to foreign antigens suggest that salamanders have the capacity for developing immune memory (Kaufman and Volk, 1994). These similarities to anurans provide reason to suspect that acquired immunity is more important to salamander parasite dynamics than is commonly assumed.

Perhaps the true importance of acquired immunity in salamanders has been overlooked as a consequence of focusing on species or parasites for which acquired immunity might be peculiarly constrained or unimportant. Neoteny, a long history of laboratory breeding and the probable origin of axolotls from an isolated population of tiger salamanders might have caused the loss of some immune functions due to developmental constraints on immunity and population bottlenecks (Bos and DeWoody, 2005). Indeed, Bos and DeWoody (2005) found high MHC-II diversity in tiger salamanders comparable to *X. laevis*, in contrast to axolotls which possess only two alleles. More recently, high MHC polymorphism has also been found in populations of the alpine newt *Mesotriton alpestris* (Babik et al., 2008). The importance of immune memory and its consequent effects on infection dynamics also vary greatly among parasite species even within the same host species (Cattadori et al., 2008), so it seems reasonable to expect that salamanders would also have significant acquired immune responses only to certain parasites. In this respect we cannot draw general conclusions about the importance of salamander immune memory based on the lack of response to one or a few parasite taxa.

To facilitate testing the general importance of immune memory in salamanders we need to identify which parasites are most likely to elicit a biologically significant immune memory response. Undertaking detailed studies of within-host responses is important, but to appreciate the consequences at the population level we need to observe population level signals. One way to detect effects of immune memory on host-parasite systems at the population level is to examine the relationship between average parasite intensity (the average number of parasites infecting individual hosts after Hudson and Dobson, 1995, but see Bush et al., 1997) and host age (Anderson and May, 1985a,b; Cattadori et al., 2005). The simplest age-intensity relationship is a constant linear increase in parasite intensity with age (Type I), expected when the rate of intrinsic parasite mortality is negligible and the infection rate is constant (Hudson and Dobson, 1995; Hudson et al., 2006). Such a linear relationship can be considered the null age-intensity relationship for encysted parasites that experience no detectable parasite mortality, which we will refer to as "persistent" parasites (Fig. 1A). The immune system might prevent establishment of persistent parasites but cannot expel them once established. For persistent parasites, acquired immunity is predicted to reduce parasite establishment in older hosts. With no mortality, this should cause age-intensity relationship to become curved, approaching an asymptote in older hosts as the rate of parasite establishment approaches zero (Type II, Fig. 1A). In contrast, parasites with significant rates of mortality (ones that the host can expel, referred to hereafter as "transient" parasites) are predicted to exhibit an age-intensity relationship that rises to an asymptote in the absence of immune memory (Type II, Fig. 1B). This asymptote is a result of parasite mortality coming to balance the number of new infections in older hosts (Hudson and Dobson, 1995; Duerr et al., 2003). For transient parasites, a Type II increase to an asymptote is the null age-intensity relationship, and acquired immune

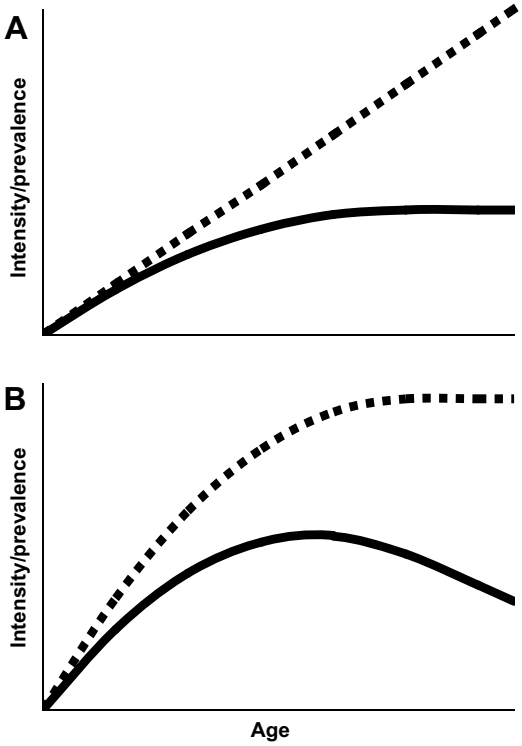

Fig. 1. Predicted effects of acquired immunity on the age-intensity relationships of (A) "persistent" parasites, which form permanent cysts and are not expelled by the host immune system, and (B) "transient" parasites, which can be expelled and are expected to have some rate of parasite mortality. Dashed curves indicate the predicted null dynamics without immune memory, and solid curves indicate predicted dynamics with significant immune memory. Any evidence of curvature to this relationship for a persistent parasite, or of decreasing infection through time for a transient parasite, potentially reflects a population-level effect of immune memory on parasite dynamics.

memory is predicted to cause this relationship to turn over and become convex (Type III, Fig. 1B) (Hudson and Dobson, 1995; Woolhouse, 1998; Cattadori et al., 2005). Any evidence of a convex age-intensity relationship (including decreasing parasite intensity with host age) for a transient parasite, or of a curved age-intensity relationship for a persistent parasite, potentially reflects an effect of immune memory on parasite infection dynamics. Similar predictions should hold for age-prevalence relationships of persistent and transient micro-parasites, for which prevalence is often a more appropriate measure than intensity. While such patterns are suggestive of acquired immune memory, it is important to assess possible alternative mechanisms which can have similar effects on age-intensity relationships (Duerr et al., 2003).

We used parasite age-intensity relationships in six populations of red-spotted newts to determine which, if any, of their parasites show evidence of eliciting a significant immune memory response strong enough to influence population-level infection dynamics. Newts live at high population densities in their aquatic adult stage and have a diverse, abundant and well-described parasite community, allowing comparisons of multiple parasite taxa in the same host population (Raffel, T.R., 2006. Drivers of seasonal infection dynamics in the parasite community of red-spotted newts (*Notophthalmus viridescens*). Ph. D. Thesis, The Pennsylvania State University, University Park, USA). In addition, newts are relatively long-lived (mean ages of 5–7.5 years in five Canadian populations, Caetano and Leclair, 1996), reducing potential impacts of seasonal fluctuations in infection on the overall shape of the age-intensity curve. We estimated the age of a subset of newts from each pond using skeletochronology and used the relationship between snout-vent length (SVL) and age to estimate the number of years adult newts had been exposed to aquatic parasites. We then examined the age-intensity relationships of 12 parasite taxa for evidence of immune memory. We found evidence of convex age-intensity relationships in three parasite taxa, two of which can be best explained by population-level effects of acquired immune memory.

2. Materials and methods

2.1. Ponds sampled

Adult newts were sampled seasonally from six ponds in and around Centre County, Pennsylvania. Two were permanent beaver ponds on stream systems, in the Penn State Experimental Forest (Mothersbaugh, "MB", N 40° 39′ 12″, W 77° 54′ 9″) and in Moshannon State Forest (Turtle Shell pond, "TS", N 40° 52′ 26″, W 78° 4′ 36″), and another was an artificial impoundment at Penn Roosevelt State Park (Penn Roosevelt, "PR", N 40° 43′ 36.8″, W 77° 42′ 8.3″). We also sampled a permanent but landlocked pond (Little Acre, "LA", N 40° 48′ 6″, W 77° 56′ 37″) and two semi-permanent (drying some winters) landlocked ponds (Mystery Newt pond, "MN", N 40° 45′ 53″, W 78° 0′ 49″; Twin Pond, "TP", 40° 46′ 49.1″, W 78° 0′ 13.9″) in the Scotia Barrens (PA State Game Lands #176). Seasonal surveys and collections of newts for dissection were conducted from 2003 and 2004 in the early spring (20 March–30 April), summer (16 July–17 August), autumn (25 September–5 November) and winter (14 January–27 February). Newts could not be obtained in winter from MN or TP due to the ponds drying. A late spring (26 May–18 June) survey was added in 2004 and replicated in 2005 following observations of differences in newt ecology between early and late spring. PR was surveyed only in June 2004.

2.2. Surveys

During each survey, meter-long sweeps of a dip net (30 × 60 cm aperture, 3 mm mesh) were taken at regular four-step intervals in a sinusoidal pattern going out to a depth of 0.5 m around the entire pond perimeter. Sex, SVL, visible signs of *Ichthyophonus* sp. infection (as described by Raffel et al., 2007b), the number of visible *Clinostomum* sp. metacercariae (described by Converse and Green, 2005) and the presence of visible cysts of the fungus-like mesomycetozoan *Amphibiocystidium viridescens* (Raffel et al., 2007a) were recorded for each individual newt. After completion of each sweep-survey, 10 newts were collected from each pond for blood collection and necropsy. Newts were transported to the laboratory in 250 mL Nalgene containers filled with pond water, anesthetized by rubbing a drop of 10% benzocaine (Oragel®) on the head, and euthanized by decapitation within 3 h of initial capture.

2.3. Parasite identification and enumeration

Trypanosoma diemyctyli (Tobey, 1906) infection was determined from blood smears. We collected blood from euthanized newts with a heparinized capillary tube and smeared a drop on a glass microscope slide. Slides were stained using the modified Giemsa staining procedure of Raffel et al. (2007b). Cells were counted at 400× magnification starting in the upper left corner of the smear and working across the slide in a standardized search pattern, moving the objective 2 mm between fields and counting all cells in each field until the erythrocyte count reached 5,000. Newts were considered infected if at least one trypanosome was observed per 5,000 erythrocytes. Although it is possible to miss low-level infections using this method, it has been sufficient to detect 100% prevalence in some populations of red-spotted newts (Raffel et al., 2007b).

Newts were surface-sterilized with 70% ethanol and a 30 mg liver sample removed for bacterial culture using a sterile technique. An equal volume of sterile PBS was added to the liver sample and homogenized using a pellet pestle (Kontes Glass Co., Vineland, NJ), after which 25 μL of homogenate was spread onto trypticase soy agar (TSA) containing 5% sheep's blood (Remel Inc., Lenexa, KS). Plates were incubated aerobically for 5 days at room temperature and visible colonies counted. The total number of colony-forming units on the plate (CFU per 12.5 mg liver) will be referred to as the bacterial load for a given newt.

The remainder of the specimen was preserved in 70% ethanol for further parasitological examination. The pleuroperitoneal cavity, internal organs and digestive tract were examined for helminth parasites. The helminths were counted and stored in a solution of 10% glycerol, 70% ethanol and 20% water. Five *Clinostomum* sp. metacercariae and three *Spiroxys* sp. nematodes were excysted as live worms for identification purposes, the former mechanically and the latter using an acid pepsin digest (Fried and Roth, 1974). Nematodes were gradually transferred to 100% glycerol by overnight evaporation and examined by light microscopy. All trematodes and acanthocephalans were stained overnight in Ehrlich's hematoxylin stain, destained in acid alcohol and gradually transferred to 100% ethanol by four 30-min steps in 80, 90, 95 and 100% ethanol. They were cleared in methyl salicylate and mounted in balsam. Helminths were identified according to the keys of Schell (1970) and Anderson et al. (1974), and from original descriptions in the primary literature (Mueller, 1932; Hopkins, 1933; Hedrick, 1935; Owen, 1946; Cheng, 1958; Uglem and Larson, 1969; Moravec, 1986). All helminths were recorded as numbers of worms per newt. Voucher specimens are deposited in the U.S. National Parasite Collection (Beltsville, Maryland) for the gastro-intestinal nematode *Amphibiocapillaria tritonispunctati* (USNPC 100739–100740), the gastro-intestinal adult trematodes *Brachycoelium* spp. (USNPC 100730, 100732, 100734, 100736) and *Plagitura salamandra* (USNPC 100728, 100729, 100733, 100735), the gastro-intestinal acanthocephalan *Neoechinorhynchus saginatus* (USNPC 100727), metacercariae of the trematode *Clinostomum* sp. excysted from muscle tissue (USNPC 100724), larval *Spiroxys contortus*

nematodes excysted from the stomach lining (USNPC 100741–100742), an unidentified species of larval nematode from cysts in the pancreas (USNPC 100743–100744), an unidentified species of digenetic trematode metacercariae from the liver, kidneys and associated mesenteries (USNPC 100745-100746), blood smears containing the protist parasite *T. diemyctyli* (USNPC 98171–98177), sections of muscle tissue infected with the mesomycetozoan protist *Ichthyophonus* sp. (USNPC 98177–98181) and sections of tissue containing subcutaneous cysts of *A. viridescens* (USNPC 99608–99619).

2.4. Bacterial identification

If at least 10 colonies of the same morphology were observed on a single plate, the isolate was sub-cultured onto TSA plates, then frozen at −80°C in 1:1 Trypticase Soy Broth:Glycerol. Forty-five isolates recovered from salamanders from five ponds were identified by 16S rDNA sequence analysis. Briefly, DNA was extracted, amplified and sequenced according to the method of Relman et al. (1992). Sequences were obtained by Davis Sequencing (Davis, CA). Bacterial isolates were identified to the family level by comparing the 16S rDNA sequences (GenBank Accession Nos. EU626739–EU626783) to a large sample of known bacterial isolates compiled by Santoni and Romano-Spica (2006), using their online program available at http://www.bioigene.it/cgi-bin/dzip/index.cgi.

2.5. Skeletochronological age estimates

The rate at which newts grow slows with age and can differ between populations (Caetano and Leclair, 1996), making it important to determine the shape of the age-length relationship in these populations. Absolute ages were estimated for a subset of newts from each pond (22, 28, 5, 5, 5 and 5 for LA, MB, MN, PR, TP and TS, respectively) using skeletochronology, the preferred method for estimating age in amphibians in the absence of accurate mark-recapture data (Smirina, 1994). These sample sizes were large enough to determine the shape of the age-size relationship for LA and MB and to test for differences in the overall age-size relationship among the six ponds. Newts and other temperate amphibians have slower bone growth during winter, resulting in lines of arrested growth (LAGs) in the periosteal bone which can be counted to determine the animal's age (Caetano and Leclair, 1996). We removed both femurs, which were then fixed in formalin for 24 h, rinsed in deionized water for 24 h, decalcified in 5% nitric acid for 15–30 min, rinsed in water again for 24 h and stained in 15% Ehrlich's hematoxylin for 24 h. We then produced 15 μm cross-sections with a freezing microtome (Cryostat Cryotome E110, Thermo Fisher, Kalamazoo, MI). We analyzed the section with the most visible LAGs between 38% and 42% of the bone length from the proximal end of the femur, within which we consistently found the maximum number of LAGs for each bone.

Skeletochronology is subject to several limitations, the most challenging of which is that inner layers of periosteal bone are resorbed during the process of bone growth and marrow enlargement (Smirina, 1994). All of the newt femurs in this study exhibited evidence of bone resorption (i.e., an enlarged marrow cavity and incomplete inner LAGs), possibly due to low calcium levels in these ponds (Raffel et al., 2007b). To estimate the number of missing LAGs, if any, for each newt, we used morphometrics to compare the size of each newt's innermost LAG to similarly sized LAGs of a young juvenile newt collected near Little Acre, as described in Fig. 2. Most (80%) newts were estimated to be missing four or fewer LAGs, with a maximum of eight LAGs missing in one very large bone section. The age ranges resulting from this procedure were similar to those found previously in red-spotted newt populations (Fig. 3A, Caetano and Leclair, 1996).

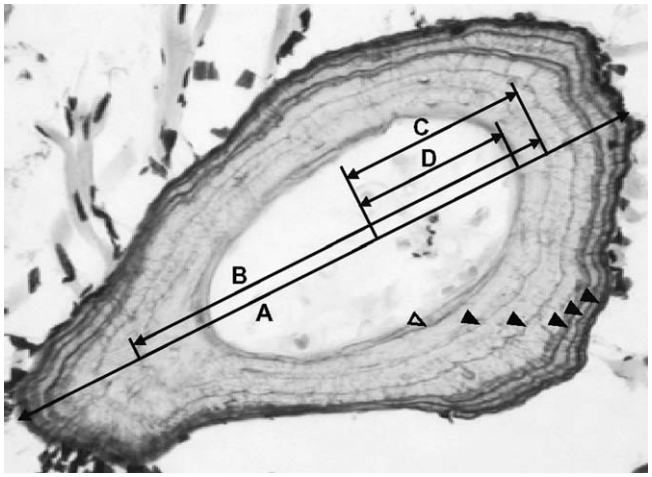

Fig. 2. Representative section of a newt femur, showing five complete lines of arrested growth (LAGs, filled arrowheads) and an inner LAG which is incomplete due to endosteal resorption (open arrowhead). Double-headed arrows indicate: (A) the long axis of the section starting from the narrow end and dividing the cross-sectional area in half, (B) the length of the innermost complete LAG along this axis, (C) the distance from the blunt end of the innermost complete LAG to the bone's approximate centre (42% of length B, based on the position of the centre of the inner LAG for the newt with the smallest complete inner LAG relative to femur diameter), and (D) the distance from the bone's approximate centre to the inner edge of the periosteal bone. The number of inner LAGs missing due to endosteal resorption was estimated based on a comparison of length D with the position of LAGs of a young juvenile newt from Little Acre. For newts whose length D was greater than the largest LAG of this juvenile newt, we estimated the number of additional missing LAGs using average measurements from six Little Acre newts whose inner LAGs corresponded to the juvenile newt's outer LAGs.

2.6. Age estimation from SVL

Newt ages for the larger parasitological dataset were estimated from SVL based on the relationship between estimated age and SVL in the skeletochronology dataset (Fig. 3A). SVL has an approximately logarithmic relationship with age in adult red-spotted newts (Caetano and Leclair, 1996), so we fitted SVL to the log-transformed skeletochronologically-derived age estimates using a general linear model blocked by pond and by sex. There was a significant main effect of pond ($F_{1,62} = 2.49$, $P = 0.041$) but no significant effects of sex or of a pond by log-age interaction (both $P > 0.2$). We then used the model fit to back-calculate newt ages from SVL in the larger dataset. Size variation and the decreasing slope of the age-length relationship led to some estimated ages which seemed unreasonably high based on the maximum skeletochronologically-derived age estimates of 14 and 13 found by us (Fig. 3A) and by Caetano and Leclair (1996), respectively, even considering that our sample size for measured individuals was much larger and therefore might have included rare older individuals. To avoid creation of spurious influential outliers in our analysis we set a maximum age of 20 years. The assumption of a logarithmic age-length relationship yielded age distributions with an early peak at 4–6 years of age followed by a gradual decrease in abundance for older newts (Fig. 3B). We repeated the age-intensity analyses assuming a linear relationship between SVL and age but without imposing limits on the maximum age, to determine whether the results were robust for the type of age estimation procedure.

2.7. Estimation of the aquatic exposure period

Most parasites of red-spotted newts infect them in the water, so the most relevant age parameter for this study was the time since a newt first became an aquatic adult, which we will refer to as the

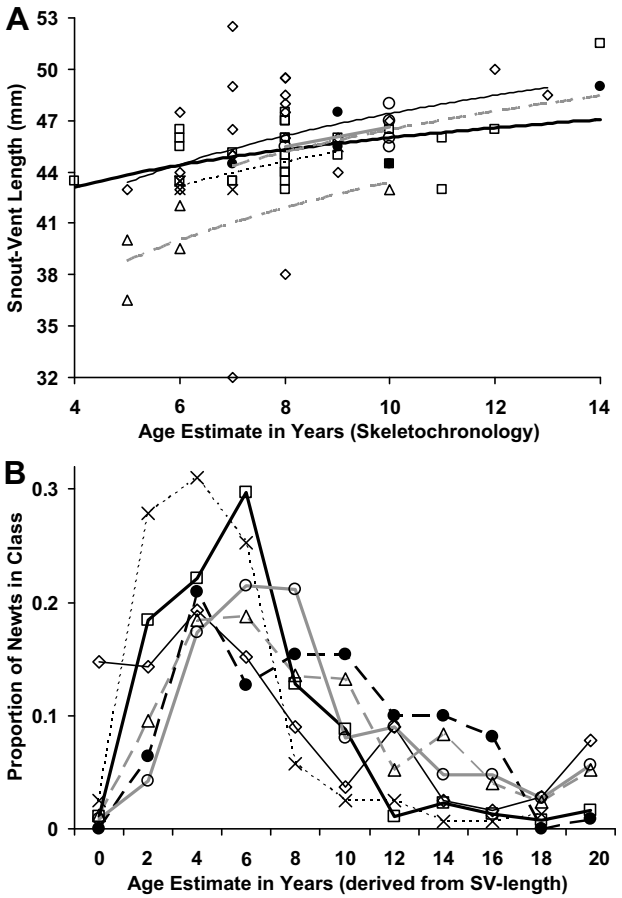

Fig. 3. Results of the age analyses, showing (A) the relationship between snout-vent length (SVL) and estimated age for a subset of newts based on skeletochronology and (B) the distributions of ages in the six newt populations as estimated from the relationship between age and SVL. Curves in A indicate the best-fit logarithmic relationship between SVL and estimated age for each pond. LA, Little Acre; MB, Mothersbaugh; PR, Penn Roosevelt; TP, Twin Pond; TS, Turtle Shell.

aquatic exposure period (AEP). Because the length of the terrestrial juvenile period can differ among newt populations (Healy, 1974), we corrected for among-pond differences in the average age at first reproduction in order to obtain estimates of the AEP for each newt. This correction would not have been necessary if we were only testing for linear effects of age on infection prevalence or intensity, but using raw ages might have made it difficult to detect quadratic effects if the average age at first reproduction differed significantly between ponds. We used the average estimated age of the smallest 40% of newts in each pond as a proxy for average age at first reproduction, and we calculated the AEP by subtracting this value from each newt's age estimate. Newts with negative estimated AEPs were assumed to be first-year breeders (exposure time = 0). To determine the sensitivity of our results to this assumption, these analyses were repeated using the smallest 20, 30, 50 and 60% of adult newts to estimate the average age at first reproduction in each pond, as well as using raw age estimates and SVL.

2.8. Statistics

We used R statistical software for all analyses (www.r-project.org). The analysis for each parasite taxon was restricted to ponds with a minimum of four newts infected by the given parasite. We used generalized linear models (GLZM) to test for significant linear and quadratic effects of the aquatic exposure period on parasite infection intensity or prevalence. All analyses were blocked to control for main effects of pond and season (i.e., the time of year at which newts were collected). We also tested the significance of a pond × AEP interaction term, which would indicate that the age-intensity relationships differed between ponds. The quadratic and pond × AEP interaction terms were removed from final models if they did not significantly improve the model fit (P-value > 0.05 based on sub-model deviance tests). Count data for helminths and bacterial load were analyzed using the negative binomial error distribution (glm.nb in package "MASS") and log-transformed for representation in figures. Prevalence data for the other microparasites (*Ichthyophonus* sp., *T. diemyctyli* and *A. viridescens*) were analyzed using a binomial error distribution. Prevalence of larval nematodes encysted in the pancreas was also analyzed using a binomial error distribution because the worm distribution was too highly aggregated to fit the negative binomial (there were either many worms encysted in the pancreas or none at all). However, age-prevalence data provides limited insight into acquired immunity for a persistent parasite, since newts can only enter the "infected" category once and all new infections occur in previously unexposed individuals. We therefore ran alternate analyses of intensity for *Ichthyophonus* sp. (proportion body covered) and larval pancreas nematodes (worm counts) using multivariate permutation tests (adonis in package "vegan" of R statistical software), testing for a quadratic effect of AEP using 100 permutations.

Small sample sizes can lead to an underestimation of mean intensity when the parasite population is aggregated within the host population (Gregory and Woolhouse, 1993), so the decreasing sample size of newts with age could bias the results of the negative binomial regressions towards negative slopes or convexity. This problem is sometimes referred to as data frailty. For this reason, we followed up the analysis of bacterial load with a bootstrap simulation analysis, to determine whether the slope of this age-intensity relationship was more negative than expected under the null model of no change in bacterial load with age. We generated new bacterial colony counts for all individuals in the analysis, selecting randomly from a negative binomial distribution with a mean equal to the average colony count of young newts (AEP < 3 years) and theta estimated from the original negative binomial GLZM analysis of bacterial load. We then re-ran the negative binomial GLZM using the new colony counts and repeated the procedure 10,000 times, to determine the proportion of randomly generated datasets with a slope as negative as the slope of the observed age-intensity relationship.

3. Results

Sufficient data were available to analyze the age-intensity profiles for 12 parasite taxa: four trematodes, three nematodes, one acanthocephalan, two mesomycetozoans, one trypanosome and bacteria in the liver. Of these, only three showed evidence of a turn over in the age-intensity profile, with decreasing parasite intensity in older newts (Table 1). *Amphibiocystidium viridescens* prevalence decreased significantly with AEP (Fig. 4B; Table 1). Bacterial load also decreased significantly with AEP, a pattern which occurred in all five ponds despite a significant pond-by-AEP interaction (Fig. 4A; Table 1), indicating among-pond differences in the rate of decrease. Bootstrap analysis showed that this finding of a decrease in bacterial load with AEP was not due to a low sample size of older newts, with none of the 10,000 simulations producing as negative a slope as the observed relationship (P < 0.0001). The bacterial load results were robust to the inclusion of two outliers (young newts with high bacterial loads, >1000 colonies), which actually strengthened the effects of AEP and pond × AEP when added back into the analysis (both P < 0.001). Bacterial isolates belonged to 12 families of bacteria, with more than 50% of isolates belonging to two families, the Enterobacteriaceae and Caulobacter-

Table 1
Results of age-intensity analyses for the twelve parasite taxa, using aquatic exposure period (AEP), calculated from snout-vent length, as a proxy for age

Response	Predictor	Coef.	ΔDev	d.f.	*P*
Clinostomum[a]	Season		42.6	4	<0.001
n = 1861 (MB)	AEP	0.061	7.9	1	0.005
Ichthyophonus[b]	Pond		26.7	4	<0.001
n = 2,708	Season		23.1	4	<0.001
(LA, MB, PR, TP, TS)	AEP	0.019	34.6	1	<0.001
Metacercariae[a]	Pond	−2.523	79.4	1	<0.001
n = 183	Season		22.1	4	<0.001
(MB, TS)	AEP	0.158	5.5	1	0.019
	Pond:AEP	−0.218	10.4	1	0.001
Pancreas nematode[b]	Season		8.6	4	0.071
n = 116 (MB)	AEP	0.115	6.3	1	0.012
Plagitura[a]	Pond		19.4	2	<0.001
n = 275	Season		14.3	4	0.006
(LA, MB, TS)	AEP		1.0	1	0.325
Spiroxys[a]	Pond		410.7	4	<0.001
n = 392	Season		13.9	4	0.008
(LA, MB, MN, TP, TS)	AEP	0.095	27.4	1	<0.001
Amphibiocapillaria[a]	Pond		181.5	4	<0.001
n = 379	Season		10.05	4	0.040
(LA, MB, MN, TP, TS)	AEP	0.022	3.3	1	0.068
Neoechinorhynchus[a]	Season		33.5	4	<0.001
n = 116 (MB)	AEP		0.9	1	0.331
Trypanosoma[b]	Pond		48.8	2	<0.001
n = 277	Season		18.5	4	<0.001
(LA, MB, TS)	AEP		1.5	1	0.223
Brachycoelium[a]	Pond		12.6	4	0.013
n = 380	Season		14.8	4	0.005
(LA, MB, MN, TP, TS)	AEP	−0.131	3.7	1	0.054
	$(AEP)^2$	−0.032	7.7	1	0.006
	Pond:AEP		33.8	4	<0.001
Bacterial load[a]	Pond		8.8	4	0.070
n = 382	Season		48.5	4	<0.001
(LA, MB, MN, TP, TS)	AEP	−0.169	13.1	1	<0.001
	Pond:AEP		11.1	4	0.030
Amphibiocystidium[b]	Season		18.3	4	0.001
n = 244 (LA)	AEP	−1.903	24.4	1	<0.001

Sample sizes (*n*) differ between analyses depending upon whether dissection was necessary for parasite detection and which ponds had sufficient infection prevalence to be included in the analysis.
LA, Little Acre; MB, Mothersbaugh; PR, Penn Roosevelt; TP, Twin Pond; TS, Turtle Shell.
[a] Negative binomial error distribution.
[b] Binomial error distribution.

aceae (Table 2). There was a significant quadratic effect of AEP on *Brachycoelium salamandrae* infection intensity, in addition to a significant pond-by-AEP interaction (Table 1), with convex age-intensity relationships in four of five ponds and a monotonic decrease in parasite intensity in the fifth (Fig. 5A).

Of the nine remaining parasites, five were encysted parasites and appear to have been persistent infections (i.e., no significant rate of detectable parasite mortality): *Clinostomum* sp. metacercariae in the muscle, an unidentified species of trematode metacercaria in the liver and kidney, *S. contortus* larvae encysted in the stomach wall, unidentified species of nematode larvae in the pancreas, and *Ichthyophonus* sp. in the musculature. All five of these parasites increased linearly with aquatic exposure time (Fig. 5B–F; Table 1). There was a significant pond-by-AEP interaction for the unidentified metacercariae, consistent with a greater rate of infection in MB than in TS (Fig. 5D, Table 1). None of the persistent parasites showed evidence of curvature in their age-intensity relationships (all quadratic terms with $P > 0.05$).

The four remaining parasite taxa (*P. salamandra*, *A. tritonispunctati*, *N. saginatus*, and *T. diemyctyli*) live in the intestinal lumen or bloodstream and can be expected to have some significant rate of intrinsic parasite mortality. None of these parasites showed any significant linear or quadratic effects of aquatic exposure time on their infection prevalence or intensity (Table 1, Fig. 4C–F), though *A. tritonispunctati* showed a non-significant trend for increasing intensity with AEP (Table 1). Results of all the parasite analyses were robust to the age estimation procedure, giving qualitatively identical results regardless of the proportion of newts used to estimate the average age at first reproduction or whether SVL was assumed to have a logarithmic or linear relationship with newt age.

Most newts had infections with multiple parasites, with 71% of the dissected newts infected by at least two different parasite taxa and a maximum number of nine concurrent infections in a single newt (counting bacterial infections if bacterial load was greater than 10 colonies). This study represents a new host record for *N. saginatus*, which has previously been reported only from creek chubs (Muzzall and Bullock, 1978).

4. Discussion

Only three of the 12 parasite taxa, bacterial load, *A. viridescens* and *Brachycoelium* spp., exhibited evidence of decreasing infection rates in older newts, as reflected by a negative slopes of the age-intensity/prevalence relationships for the first two and a convex age-intensity profile consistent in all five sites for the latter. The null expectation for all three parasites was an increase in prevalence or intensity to an asymptote in relation to aquatic exposure period, because we expected all three to exhibit significant parasite mortality (i.e., infections are transient). Bacterial load and *A. viridescens* both reached their peak infection levels in young newts, suggesting either very high infection rates in the first year or two after newts return to the aquatic habitat to breed or infections which occurred prior to adult newts returning to the pond. These patterns of decreasing infection prevalence or intensity with age were consistent with the predicted effects of immune memory on the infection dynamics of these parasites. However, acquired immunity is only one of several mechanisms with the potential to produce convex or negative age-intensity relationships.

One likely explanation of the convex age-intensity relationship for *Brachycoelium* spp. is decreasing exposure of newts to this parasite taxon with age, as suggested by Jackson and Beaudoin (1967) to explain a similar pattern of *Brachycoelium occulatum* infection in juvenile and adult newts. Unlike all other parasites in this study, *Brachycoelium* spp. utilize a terrestrial snail as an intermediate host, so infection only occurs when newts ingest snails on land (Cheng, 1960). Although adult newts do return to land periodically, especially during the summer (Sever, 2006), their exposure to these parasites should be reduced compared with fully terrestrial juveniles. Younger adult newts might still spend more time on land than older adults, accounting for *Brachycoelium* spp. intensity continuing to increase for the first few years of adulthood. This explanation is unlikely to account for decreasing parasitic intensity with age for predominantly aquatic parasites like bacterial pathogens or *A. viridescens*.

Considering the number of newts infected with multiple parasite taxa, parasite age-intensity relationships might also be influenced by direct or immune-mediated parasite competition. A negative interaction between two parasites, in which infection by one parasite leads to exclusion of another parasite, might cause a convex age-intensity relationship if the former parasite increased in intensity with age. *Brachycoelium* spp. was the only parasite taxon in this dataset whose dynamics appear to have been influenced by an interaction with another parasite, the unidentified trematode metacercariae from the liver (Raffel, T.R., 2006. Drivers of seasonal infection dynamics in the parasite community of red-spotted newts (*Notophthalmus viridescens*). Ph. D. Thesis, The Pennsylvania State University, University Park, USA); however, this interaction is unlikely to have driven the observed age-intensity relationships

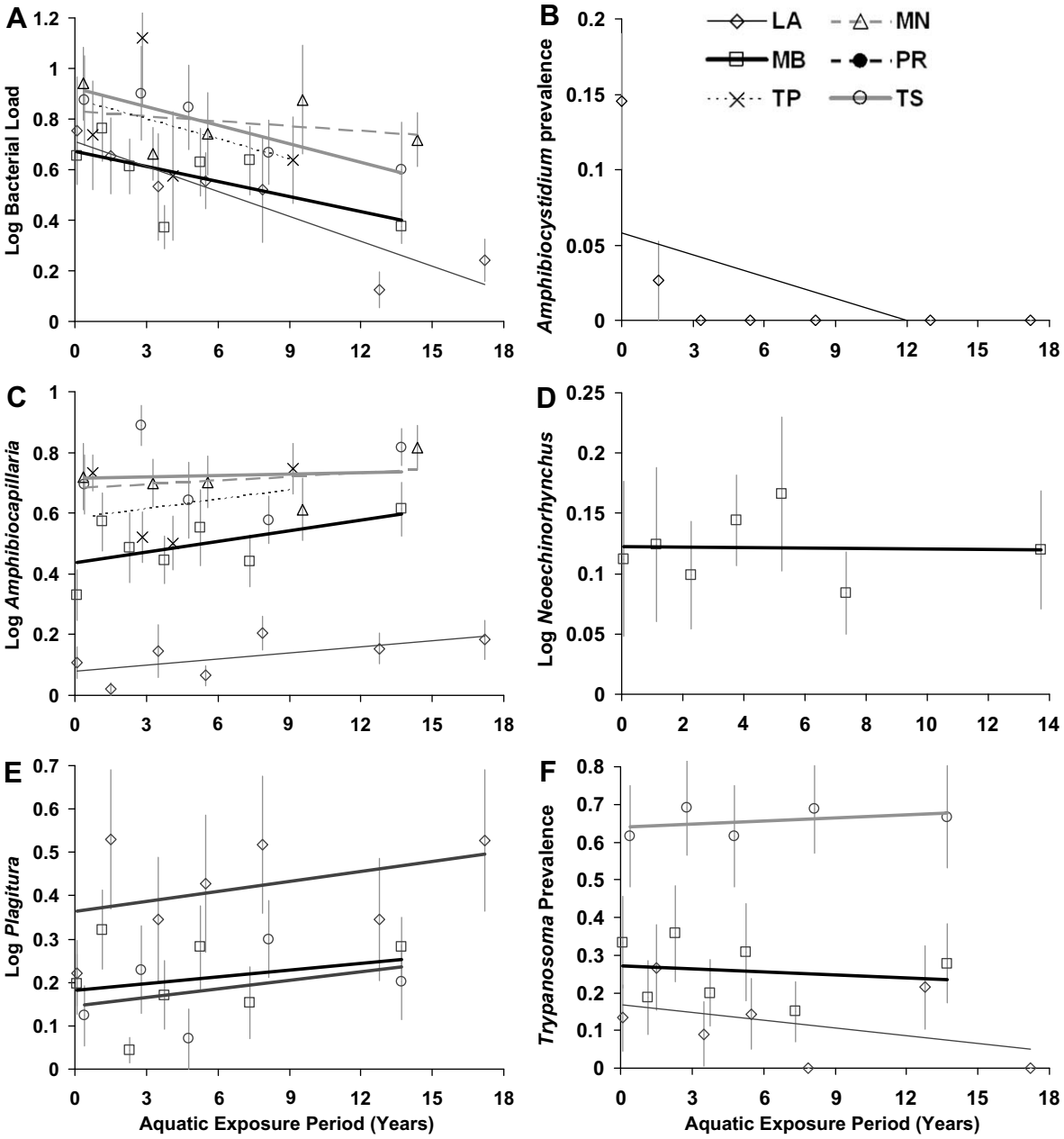

Fig. 4. Relationships between estimated aquatic exposure period (number of years since a newt started breeding) and prevalence or intensity of six transient parasite taxa of red-spotted newts: (A) numbers of bacterial colony-forming units in the newt liver, (B) *Amphibiocystidium viridescens*, (C) *Amphibocapillaria tritonispunctati*, (D) *Neoechinorhynchus saginatus*, (E) *Plagitura salamandra* and (F) *Ttrypanosoma diemyctyli*. For display purposes, newts were separated into age classes for each pond based on sample size (approximately 15 dissected newts per age category), so that each point represents the average age and intensity/prevalence for newts in a single age class. Parasite counts were log-transformed to improve normality. Error bars indicate standard errors. LA, Little Acre; MB, Mothersbaugh; PR, Penn Roosevelt; TP, Twin Pond; TS, Turtle Shell.

because these metacercariae were only observed in two of the five ponds.

Other possible drivers of convex age-intensity relationships for bacteria and *A. viridescens* are an increase in innate immune response with age or parasite-induced host mortality (Duerr et al., 2003), but neither seems a likely driver in this study. Although larval amphibians do improve in immune responsiveness as they approach adulthood (Rollins-Smith, 1998), the scarce data on age-dependent immunity in adult amphibians suggests if anything that the immune system declines with age in adult amphibians (Torroba and Zapata, 2003), contrary to this hypothesis. Parasite-induced host mortality can lead to a convex age-intensity relationship when coupled with an aggregated parasite distribution, because older individuals with heavy parasite burdens are expected to die at a greater rate (Duerr et al., 2003). In this study, both bacterial load and *A. viridescens* had aggregated distributions (variance-to-mean ratios of 127.3 and 132.8, respectively). Experimental infections by opportunistic bacterial pathogens in the laboratory are usually sub-lethal in amphibians (e.g., Brodkin et al., 1992; Maniero and Carey, 1997; T.R. Raffel, unpublished data), although other factors can interact with these infections to induce mortality (Taylor et al., 1999). *Amphibiocystidium viridescens* appears to cause significant newt mortality, at least in recently shipped and probably stressed captive newts (Raffel et al., 2007a).

Table 2
Bacterial isolates from livers of wild-caught newts

Bacterial family	# Isolates	General distribution	Normal amphibian microflora
Enterobacteriaceae	14	Water, soil, intestines[a]	Frog intestines[b], newt skin[c]
Caulobacteraceae	10	Water[d]	
Xanthomonadaceae	6	Water, soil[e]	Salamander skin[f]
Microbacteriaceae	3	Water, soil[g]	Frog[c] and salamander[c,h] skin
Micrococcaceae	3	Skin[i]	Salamander skin[h]
Oxalobacteraceae	2	Water, intestines[j]	Salamander skin[h]
Pseudomonadaceae	2	Water, moist soil[k]	Frog[c], salamander[c,f,h] and newt[c] skin
Aeromonadaceae	1	Water[l]	Newt skin[c], frog intestines[b]
Enterococcaceae	1	Water, soil, intestines[m]	
Flavobacteriaceae	1	Water, soil[n]	Salamander skin[c,h]
Sphingobacteriaceae	1	Water/soil[o]	Salamander skin[f]
Sphingomonadaceae	1	Water[p]	

The isolates are grouped into 12 families of common environmental bacteria, most of which have been found associated with amphibian skin surfaces and/or intestinal contents.

[a] Farmer (1999).
[b] Hird (1983).
[c] Culp et al. (2007).
[d] Tsang et al. (2006).
[e] LaSala et al. (2007).
[f] Harris et al. (2006).
[g] Funke and Bernard (1999).
[h] Lauer et al. (2007).
[i] Kloos and Bannerman (1999).
[j] Stewart et al. (2004).
[k] Kiska and Gilligan (1999).
[l] Altwegg (1999).
[m] Facklam et al. (1999).
[n] Bernardet et al. (1996).
[o] Steyn et al. (1998).
[p] Schreckenberger and von Graevenitz (1999).

Whether or not these parasites cause mortality, infections by both parasite taxa appear to be very short in duration, with either host mortality or clearance of the infection occurring within a few weeks (Raffel et al., 2007a and unpublished data). Therefore the observed infections were probably acquired within a month of sampling rather than accumulated over the newts' lifespans. This time scale of infection is too short for parasite-induced host mortality to play a significant role in shaping an age-intensity relationship spanning many years, making parasite-induced mortality unlikely to have caused decreased parasitism in older newts. Experiments will be necessary to determine whether these parasites influence newt survival in natural ponds.

The four other parasite species expected to have significant parasite mortality rates were *T. diemyctyli*, *P. salamandra*, *N. saginatus* and *A. tritonispunctati*. None of these four parasites exhibited a significant change in intensity with increasing aquatic exposure period, indicating that infection rate was equal to the parasite mortality rate (Fig. 4C–F). Like bacterial load and *A. viridescens*, these parasites appeared to reach their equilibrium infection intensities within a few years of the newts returning to the ponds. This result suggests high infection rates during those first few years. However, non-zero infection levels in young newts could also be caused by infections carried over from the larval period. Terrestrial juvenile newts have been found to harbour several of the aquatic parasites observed in this study, including *T. diemyctyli*, *A. tritonispunctati*, *P. salamandra* and *S. contortus* (Fischthal, 1955; Jackson and Beaudoin, 1967; Gill, 1978). This also helps to explain why many of the persistent parasites also had non-zero parasite intensities in young newts (Fig. 5B–F), although size variation and the subsequent lack of precision in age estimates probably also contributes to this pattern.

None of the five encysted "persistent" parasites showed any evidence of curvature in their observed age-intensity relationships, indicating no decrease in infection rate with age that could be attributable to the effect of immune memory. The shapes of the true underlying age-intensity curves for these parasites remain unknown because we did not distinguish live from dead encysted parasites. Parasitic cysts typically remain intact even if the encysted worm dies (Martin and Conn, 1990), making the observed rate of parasite mortality zero whether or not the parasites themselves were alive. Immune memory should still have led to curved age-intensity relationships of these parasites, provided that it decreased the rate of parasite establishment. We cannot entirely rule out increased parasite mortality due to acquired immunity, though such an effect seems likely to correlate with effects on establishment, which should have been detectable. Complete data on the status of encysted parasites or manipulative experiments would be necessary to draw stronger conclusions about the absence of acquired immunity against these parasites.

All of the bacterial isolates identified in this study came from families of environmental bacteria, many of which have also been found associated with amphibian skin and intestinal contents (Table 2). It therefore seems reasonable to suppose that most of the observed infections by bacteria in this study were opportunistic infections by environmental bacteria or members of the normal microbial flora, as suggested by Jacobson (1984) and Shotts (1984) regarding infections of amphibians by *Pseudomonas* sp. and *Aeromonas* sp., respectively. If the observed age-intensity relationships are indeed due to immune memory, the diversity of bacteria infecting newts might account for the slow rate of decrease in infection levels with age, relative to the pattern for *A. viridescens* (Fig. 4A and B). Newts might need to become exposed to many different bacterial strains before they acquire immunity to sufficient strains to experience a significant drop in bacterial infection rates in general, even if they rapidly develop immune memory to individual strains. Indeed, the pattern for bacterial load is reminiscent of the gradual decrease in incidence with age of the human common cold, which is also caused by many antigenically distinct pathogen strains (Heikkinen and Jarvinen, 2003). Alternatively, these opportunistic bacteria might have low pathogenicity, reducing the need for newts to rapidly acquire immune memory. However, being opportunistic does not necessarily imply low pathogenicity, particularly with microbes cultured from normally sterile organs such as the liver.

The two parasites exhibiting age-intensity relationships indicating possible effects of acquired immunity were notably both microparasites, i.e., organisms which undergo asexual reproduction within the host. Interestingly, studies on *X. laevis*, an anuran, have detected significant immune memory responses to both helminth and viral infections (Jackson and Tinsley, 2001; Gantress et al., 2003). Different aspects of the acquired immune response are involved in fighting off different types of parasites (Janeway et al., 2001), so it is possible that newts lack effective mechanisms for mounting immune memory responses to helminths. The data for some parasite taxa might simply have been inadequate to detect immune memory responses which were in fact present, although the sample sizes obtained in this study should have been large enough to detect most biologically significant effects. While it is possible that other processes counteract and mask the effects of acquired immunity (e.g., decreased innate immune investment in older hosts as they increase investment in reproduction), in the absence of independent evidence this conclusion is less parsimonious than the null hypothesis of no effect.

Based on our findings, at least some parasites of red-spotted newts do exhibit age-intensity relationships indicative of immune memory responses, in particular *A. viridescens* and opportunistic bacterial pathogens. However, 10 of 12 parasite taxa provided no

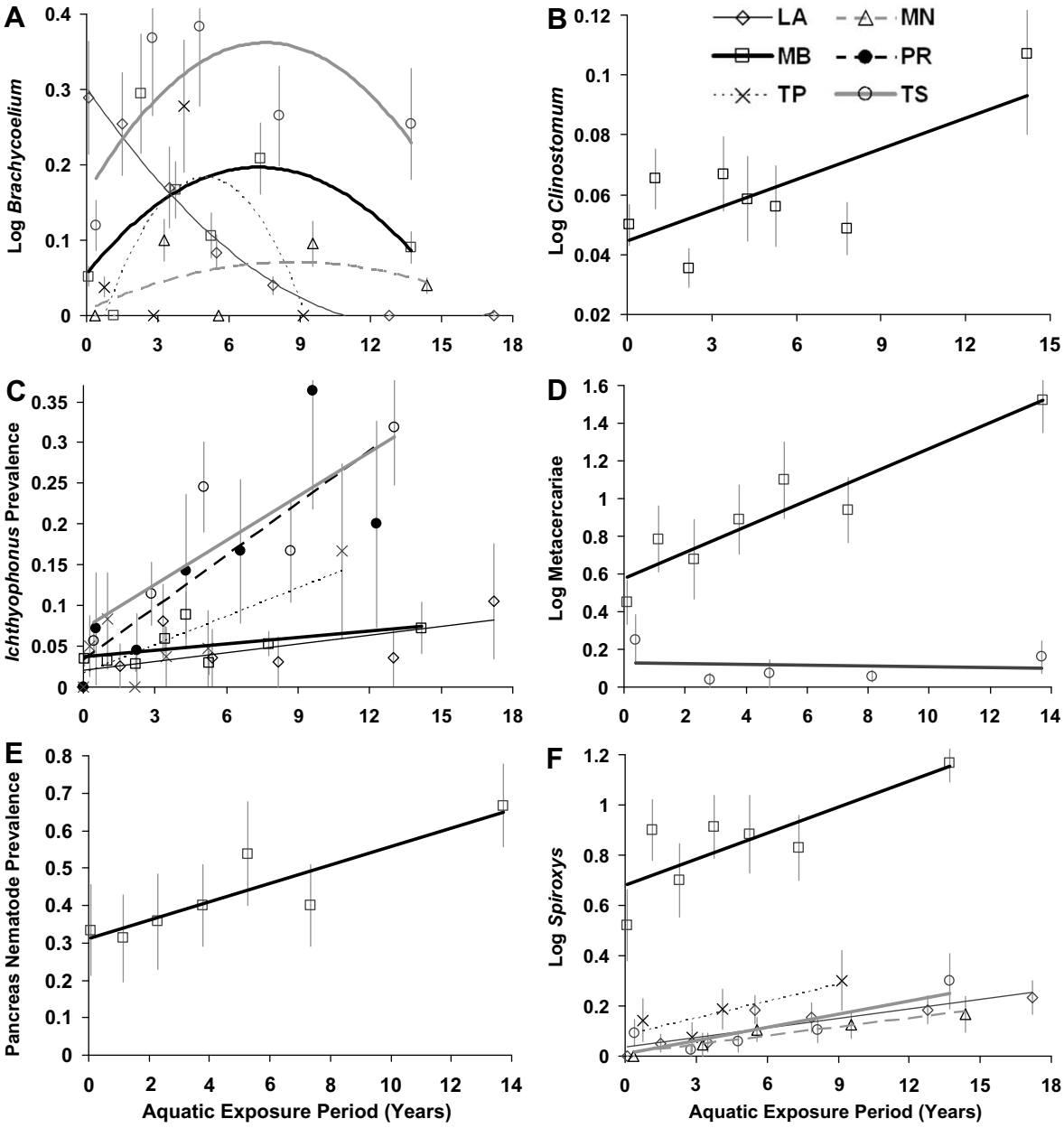

Fig. 5. Relationships between estimated aquatic exposure period (number of years since a newt started breeding) and prevalence or intensity of (A) *Brahycoelium* spp., a transient parasite taxon, and five persistent parasite taxa of red-spotted newts: (B) *Clinostomum* sp. (C) *Ichthyophonus* sp., (D) unidentified species of metacercariae encysted in the liver and kidney, (E) unidentified larval nematode species encysted in the pancreas and (F) *Spiroxys contortus*. For display purposes, newts were separated into age classes for each pond based on sample size (approximately 15 dissected newts per age category), so that each point represents the average age and intensity/prevalence for newts in a single age class. Parasite counts were log-transformed to improve normality. Error bars indicate standard errors. LA, Little Acre; MB, Mothersbaugh; PR, Penn Roosevelt; TP, Twin Pond; TS, Turtle Shell.

evidence of immune memory, highlighting the importance of investigating multiple parasites to avoid missing those for which acquired immunity is important. It will be necessary to use experimentation to definitively exclude competing explanations of these age-intensity relationships, such as parasite-induced host mortality, and to determine whether acquired immunity is necessary and sufficient to explain the observed patterns. Since infections by *Amphibiocystidium* spp. have yet to be induced experimentally, bacterial pathogens provide the best candidate parasite taxon for such tests. However, the alternative hypotheses seem unlikely to account for the observed patterns. Our results suggest that immune memory has important and consistent effects on newt parasite dynamics under natural conditions, at least for some of their parasites.

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