

**Environmental constraints influencing survival of an African parasite in a north temperate habitat: effects of temperature on egg development.**

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## SUMMARY

Factors affecting survival of parasites introduced to new geographical regions include changes in environmental temperature. *Protopolystoma xenopodis* is a monogenean introduced with the amphibian *Xenopus laevis* from South Africa to Wales (probably in the 1960s) where low water temperatures impose major constraints on life cycle processes. Effects were quantified by maintenance of eggs from natural infections in Wales under controlled conditions at 10, 12, 15, 18, 20 and 25°C. The threshold for egg viability/ development was 15°C. Mean times to hatching were 22 days at 25°C, 32 days at 20°C, extending to 66 days at 15°C. Field temperature records provided calibration of transmission schedules. Although egg production continues year-round, all eggs produced from early August to late April will die without hatching. Output contributing significantly to transmission is restricted to 10 weeks (May–mid July). Host infection, beginning after a time lag of 8 weeks for egg development, is also restricted to 10 weeks, July–September. Habitat temperatures (mean 15.5°C for June–August in 2008) allow only a narrow margin for life cycle progress: even small temperature increases, predicted with ‘global warming’, enhance infection. This system provides empirical data on the metrics of transmission permitting long-term persistence of isolated parasite populations in limiting environments.

Key words: parasite introductions, temperature, Monogenea, *Protopolystoma*, egg development, *Xenopus*, global warming,

## INTRODUCTION

The introduction of parasites into new geographical regions can have dramatic ecological impacts, facilitate host switches, threaten biodiversity and increase disease (Kennedy, 1994; Taraschewski, 2006; Dunn, 2009). Where transfers cross different climate zones, the events and adaptations required may mirror those linked with predicted trends in climate change, especially ‘global warming’ (e.g. Marcogliese, 2008). Complex interactions may occur, as in the case of *Schistosoma mansoni* and *S. intercalatum* in Cameroon where interspecies hybridization and exploitation of new intermediate hosts have changed the ecology of infection (Webster *et al.* 2003). In aquaculture, the geographical expansions of *Anguillicola crassus* and *Gyrodactylus salaris* are well-documented and include host range extension to native fish species in novel areas (Kennedy and Fitch, 1990; Bakke *et al.* 2007). Establishment of introduced species may generate intense selection pressures involving adaptation to new hosts, transmission cycles and environmental conditions (e.g. Mas-Coma *et al.* 2009).

The focus of this study is a helminth parasite of an ectotherm vertebrate host in which all life cycle stages are influenced directly by external environmental temperature. This increases the impact of novel thermal regimes in areas of introduction in contrast to parasite life cycles in endotherm hosts where only the ‘off-host’ stages are exposed to external temperature change.

The African amphibian *Xenopus laevis* has been employed worldwide in biological research since the 1930s. Associated with this laboratory use and the pet trade, the species has been released into diverse environments in North and South America, Europe and Asia (Tinsley and McCoid, 1996; Lillo *et al.* 2005; Lobos and Jaksic, 2005; Fouquet and Measey, 2006). In several regions, environmental conditions may replicate the Mediterranean climate of the South African Cape where introduced populations almost certainly originated. For instance, in California, USA, conditions are near optimal for individual and population growth (Tinsley and McCoid, 1996). However, *X. laevis* has also been introduced in regions with cooler, apparently less favourable, temperature regimes as in the UK.

*Protopolystoma xenopodis* is a monogenean specific to *X. laevis* that has become established with its host species in several novel geographical regions including one isolated colony in Wales (Jackson and Tinsley, 1998a; Tinsley and Jackson, 1998). This host population was probably introduced in the 1960s (Tinsley and McCoid, 1996; Measey and Tinsley, 1998) and, in the absence of evidence of subsequent introductions, its parasite almost certainly arrived at the same time and has persisted to the present. Strict host specificity excludes any potential transfer to other amphibian species and there are no reservoir hosts that could maintain the parasite independently of *X. laevis*.

Based on material from Africa, there is relatively comprehensive information on the life cycle characteristics of *P. xenopodis*, making this one of the best documented monogeneans with respect to environmental factors affecting reproduction, transmission and survival (reviewed by Tinsley, 2004). Parasite biology is highly sensitive to external environmental factors especially temperature (Jackson and Tinsley, 1988, 1998a, 2002), host factors especially immunity (Jackson and Tinsley, 2001, 2003, 2005), and within-population parasite factors including intra-specific

competition (Jackson and Tinsley, 1988) and inter-specific interactions (Jackson and Tinsley, 1998b, Jackson *et al.* 1998, 2006). Field and laboratory data show that *P. xenopodis* populations are characterized by relatively low prevalence (40%) and very low intensities of infection (mean 1-2 adult worms/ host, maximum burdens almost invariably <7 worms/ host), low reproductive output (typically around 9 eggs/ worm/ day at 20°C), and a pre-patent period of about 3 months at 20°C (Tinsley, 2004). Most published studies relate to parasite performance at 20°C, but more limited data confirm that constraints on life cycle dynamics are severe at lower temperatures. Thus, *per capita* egg production below 10°C is <1 e/w/d and eggs fail to complete development at low temperatures (Jackson and Tinsley, 1998a; Jackson *et al.* 2001). Even a 5°C decrease in temperature, relevant to temperate regions, may extend the time to hatching of infective larvae from about 4 weeks at 20°C to a median of about 9 weeks at 15°C (Jackson *et al.* 2001). These rate-limiting effects contribute to seasonal cycles of transmission in natural habitats in southern Africa, including the Cape where water temperatures fall to 10°C in winter and transmission is minimal for 2-3 months each year (Tinsley, 1996). This response to low temperature suggests that, in a cool north temperate climate, transmission may be entirely precluded for much longer periods annually.

The principal aim of this study is to quantify temperature effects at a series of key points in the life cycle: a) viability and developmental rates of eggs; b) growth and developmental rates of juveniles post-infection; c) period to maturity; d) survival post-infection and contribution to further transmission. This paper provides the background and presents results on stages developing in the external environment (a). A separate account records development of within-host stages (Tinsley *et al.* 2010). The approach adopted has recorded the schedules of development of life cycle stages taken from natural infections 'in the wild' and maintained under controlled environmental conditions in the laboratory. Temperature regimes were selected to permit calibration of life cycle timing in the range 10-20°C (relevant to the Welsh site) and at 25°C (relevant to the natural range in Africa and exploring the potential effects of climate warming). Overall results are of wider significance for assessing the feasibility of exotic parasite infections (in this case from Africa) to become established in cool temperate environments, and will contribute to the debate on the effects of global climate change on the potential for increased parasite infection levels and disease severity.

## MATERIALS AND METHODS

### *Fieldwork*

This investigation forms part of a larger ecological study of an introduced population of *X. laevis* in the Alun Valley, Glamorgan, Wales, continuing from 1981 to the present. Infections of *P. xenopodis* were followed in 1999 – 2008 at a single pond in pasture farmland where all *X. laevis* were individually-marked.

Water temperatures at the study site were recorded throughout the year (at 30 min intervals) with Tinytag Aquatic 2 and Tinytag Plus data loggers (Gemini Data Loggers (UK) Ltd) submerged on the pond bottom (water depth approx. 60 cm). Temperatures at other water depths, and pH, were also recorded during fieldwork visits.

### *Sources of material*

To investigate factors affecting egg development, trials in the range 10-20°C employed eggs collected from naturally-infected wild-caught *X. laevis* from the introduced population in Wales. Trials at 25°C employed eggs from patent experimental infections of lab-raised juvenile *X. laevis* previously exposed to parasite larvae hatched from eggs originating from wild-caught Welsh hosts (i.e. a laboratory F1 generation of Welsh parasites). As a guide to the genetic diversity of eggs in these trials, the wild-caught *X. laevis* comprised an 'egg factory' of 11 hosts, producing eggs at a rate indicative of 1-2 worms/ host. The lab-raised hosts comprised 13 individuals carrying an estimated 1-5 adult worms each.

### *Laboratory procedures*

Parasite eggs were pooled from aquaria in which infected *X. laevis* had been maintained at 20°C in a 12L:12D photoperiod. The water was allowed to settle and sediment transferred, with rinsings, into crystallizing dishes; eggs were transferred with a Pasteur pipette to Petri dishes and incubated at controlled temperature and illumination.

To standardize the effects of conditions prior to transfer to selected temperatures, eggs were harvested over 24h periods and collected each day at 10.00-12.00h. Water samples were decanted into crystallizing dishes and eggs transferred to 40mm diameter Petri dishes half-filled with aged, dechlorinated tapwater at 20°C. Dishes were transferred to incubators by 16.00h on the collection day and maintained in the centre of controlled environment cabinets (Sanyo Environmental Test Chamber MLR-351, Sanyo Ltd) at 10, 12, 15, 18, 20 or 25°C with 12L:12D photoperiod. Temperature variation was typically <0.1°C. At each temperature, there was a minimum of 4 dishes of eggs collected over different 24h periods. Eggs were left undisturbed in the incubators for the first 2 weeks of development (at 25°C) or 3 weeks (other temperatures) and then subjected to a standard routine of checks carried out between 09.00 and 10.00h each day. All dishes were removed from an incubator together so that eggs were disturbed, out of the controlled conditions, for the same time. Each dish was exposed to illumination from a fibre-optics light on the microscope stage generally for a maximum of 1 min. Numbers of eggs hatched since the previous check were recorded every 24h; empty egg capsules were removed after hatching. To counteract evaporation, especially at higher temperatures, Petri dishes were topped up using water aged in the incubator (all dishes at one temperature were topped up at the same time). To avoid growth of algae and potential increase in salt concentrations following topping-up, eggs were transferred to new dishes at approximately 2 week intervals: the replacement dishes of water were left in the incubator for the night preceding transfer and all dishes at a particular temperature were transferred on the same day. A further set of trials with otherwise identical manipulations employed flat-bottomed 96-well microplates (Nunc) in place of Petri dishes (following Jackson and Tinsley, 2007), but only the data recorded at 10°C were used in this account. The movements of dishes, exposure to light on the microscope stage, and disturbance of eggs were intended to provide a consistent stimulus for emergence of those larvae ready to hatch on a particular day.

### *Data analysis*

Data were analyzed using SPSS version 16. Generalized linear models with binomial error distributions were employed to investigate the influence of temperature on egg viability; AIC scores were used to assess model fits and likelihood ratio tests used to assess model significance. The influence of temperature on time to hatching was investigated non parametrically due to inequality of sample variances using Spearman's Rank and Mann-Whitney U tests. Means and percentages are given with their standard errors as estimated from models.

## RESULTS

### *Environmental conditions at the field site in Wales*

The habitat of the *X. laevis* population is a pond with area c. 140m<sup>2</sup>, constructed in the mid 1800s for livestock watering; maximum water depth is c. 60 cm overlying up to 1 m of accumulated mud. About 70% of the perimeter is formed by vertical limestone walls, but there are ramps providing gently sloping banks for access by livestock. About half of the water surface is shaded by tree canopy and about half exposed to sun. Water pH is near neutral. The pond has no drainage inflow or outflow: water derives from precipitation and groundwater seepage.

Temperatures for 2 years, 2007 and 2008, provide a reference for the field conditions experienced by the host-parasite system. In the first spring, air temperatures in April 2007 were the warmest on record for Wales (and the UK) (data since 1914) with the mean 3.3°C and maximum 4.7°C above the average for 1971-2000. However, temperatures returned to average during May and for the summer as a whole (<http://www.metoffice.gov.uk/climate/uk/2007/>). In 2008, summer mean temperatures in Wales were exactly average (<http://www.metoffice.gov.uk/climate/uk/2008/>). Fig. 1 shows water temperatures in the pond for December 2006 to December 2008. Data logger records for mean temperatures suggest that conditions were similar in the 2 years. Considering the periods of greatest activity for hosts and parasites, average temperature between 1 May and 1 October was 14.9 (range 9.6 – 21.1)°C in 2007 and 14.9 (10.1 – 19.9)°C in 2008, and between 1 June and 1 September was 16.2 (12.8 – 21.1)°C in 2007 and 15.5 (12.6 – 18.6)°C in 2008. However, these averages conceal considerable differences relevant to this study, especially in the extension of warmer temperatures both earlier and later in summer 2007. Thus, water temperatures rose to 15°C 2 weeks earlier in 2007 than in 2008 and fell below 15°C 1 week later in 2007 than in 2008. The effect is indicated by the total time logged above 15°C (including all fluctuations): 104 days in 2007 compared with 75 days in 2008, and in the total area above 15°C: 3802°C h and 1886°C h respectively. With reference to present studies of parasite developmental periods (see below), water temperatures were >12°C for 28 weeks in 2007 and 23 weeks in 2008; >15°C for 20 weeks in 2007 and 17 weeks in 2008; >18°C for 6 days in 2007 and 2 days in 2008 (Fig. 1).

Between these 2 summers, from September 2007 to May 2008, the logger record showed temperatures <12°C for 205 days and <10°C for 169 days. Water temperatures were more-or-less continuously <10°C from mid-October to late April (over 6 months) (Fig. 1). In winter, the pond was ice-covered periodically; water temperatures fluctuated between 2°C and about 10°C and were <6°C on a total of 28

days in 2006-2007 and 47 days in 2007-2008. Spot checks on diurnal temperature variation at different water depths and locations within the pond showed a range of up to 8°C at midday in early June, between deep water in permanent shade (15°C both in the water column and on the mud substrate) and shallow water over mud banks exposed to sunshine (23°C).

#### *Effects of temperature on embryo viability*

Viability was assessed as proportion of larvae completing development within the egg capsule at 10, 12, 15, 18, 20 and 25°C (individual sample sizes in Fig. 2, total 742). Embryos showed no visible signs of development at 10 and 12°C. After incubation at both temperatures for 3 months, eggs were transferred to 20°C to determine whether development would resume: all were found to have died without any embryo differentiation. Developmental success changed over a relatively narrow temperature range: the 3°C increments produced a shift from zero viability at 12°C, to 25% ( $\pm 3.1$ ) at 15°C, and 89% ( $\pm 2.4$ ) at 18°C. The further 2° increase to 20°C resulted in no improvement in egg viability (83%  $\pm 4.0$ ); 100% of eggs completed development at 25°C. The influence of temperature on egg viability was highly significant and a step function with temperature included as a cubic term produced the best fit to the data ( $\chi^2_{(df=3)}=607.5$ ;  $p \ll 0.001$ ). *Post hoc* tests (Fisher's LSD) were used to make pairwise comparisons between temperatures. With the exception of the 10-12 and 18-20°C comparisons ( $p=0.222$ ), all pairwise tests were highly significant ( $p < 0.001$ ) Oncomiracidia that completed development within the egg capsules all hatched successfully.

#### *Effects of temperature on egg hatching schedules*

Fig. 3 shows the proportions of each sample that hatched each day at 15, 18, 20 and 25°C (sample sizes 49, 149, 73, 97). Mean times to hatching were  $66.2 \pm 0.69$ ,  $39.5 \pm 0.19$ ,  $32.3 \pm 0.36$  and  $21.9 \pm 0.08$  days respectively. Increasing temperature resulted in significantly shorter hatching time (Spearman's Rank  $r_{(366)} = -0.941$ ;  $p \ll 0.001$ ). Hatching windows (the period over which larvae emerged) were clearly distinct and non-overlapping for temperatures separated by 3 and 5°C, but there was an overlap with the 2°C separation between 18 and 20°C (Fig. 3). A Mann-Whitney test demonstrated a significantly shorter period to hatching at 20°C compared with 18°C ( $U_{(149,73)} = 510.5$ ;  $p \ll 0.001$ ). The durations of hatching windows increased with declining temperature. At 25°C, hatch synchrony was high with all larvae emerging over 6 days (days 19-24) and most hatching on a single day (66% on day 22). At 20°C, the hatching window was 17 days (but 95% of emergence occurred over 12 of these days); at 18°C hatching occurred over 14 days and at 15°C over 21 days. The relationship between hatching time and temperature was non-linear: the 5°C decrease from 25 to 20°C produced a 50% increase in mean time to hatching, but the decrease from 20 to 15°C doubled the equivalent period. With further decrease to 12°C all eggs died without completing development (see above).

## DISCUSSION

### *Environmental conditions affecting infection*

In comparison with most parasitic platyhelminths, *P. xenopodis* is characterized by low prevalence and intensity in natural populations and low rates of egg production (all reducing output of infective stages into the environment), relatively long developmental period to egg hatching (delaying transmission), and slow development post-infection to maturity (extending generation time) (reviewed in Tinsley, 2004, 2005). These processes are temperature-dependent and outcomes are therefore further constrained in cool environments. From this study, correlation of water temperatures in the habitat of *X. laevis* in Wales with developmental schedules recorded in the lab emphasizes the very narrow window for activity by infective stages in the external environment. Water temperatures only briefly exceeded 18°C (for a few days each year) but parasite eggs failed to develop at temperatures <15°C that occur for about 8 months each year; infective larvae required 11 weeks to complete hatching at 15°C.

Many studies in the literature provide basic data on effects of temperature on parasite life cycles. Overall, there is extensive evidence that temperature represents a major factor regulating transmission and parasite population numbers: see, for instance, van Dijk and Morgan (2008) (parasites in endotherm hosts) and Chubb (1977) (ectotherm vertebrates). For monogeneans, there are empirical data showing the temperature dependence of specific life cycle stages (e.g. Kearns, 1986; Tubbs *et al.* 2005). Now-classic studies on helminths include the finding that development of eggs and intramolluscan stages of *Fasciola hepatica* are inhibited below 10°C (Ollerenshaw, 1974). This should preclude completion of the life cycle during periods of the year and in geographical regions where low temperatures persist for long periods. Thus, sheep liver fluke has not established in Iceland where mean temperatures remain below 10°C for over 10 months each year (Torgerson and Claxton, 1999). On the other hand, fascioliasis is a severe endemic disease in the High Andes where meteorological records show that average monthly temperatures do not rise above 7°C (Mas-Coma *et al.* 2003). The explanation lies in short-term variations in micro-environmental conditions. At high altitudes, in the air-water interface on damp soil, solar radiation may induce diurnal pulses of parasite development, ultimately allowing transmission (Mas-Coma *et al.* 2003; Tinsley, 2005; Robinson and Dalton, 2009).

The *Protopolystoma* – *Xenopus* system does not allow alternative interpretation of thermal limits based on micro-variations in temperature. Water volume and relative thermal stability in the study site pond exclude small-scale fluctuations that could improve the developmental schedule of eggs on the pond substrate. The 30 min intervals between data logger records confirm the absence of significant micro-level thermal variability (Fig. 1).

The schedule for development of eggs of *P. xenopodis* calculated in this study is likely to be robust for the majority of total output that accumulates on the pond bottom where host animals typically occur both when inactive and when feeding on benthic invertebrates (see Measey and Tinsley, 1998). By contrast, the thermal regime may be more favourable for eggs laid on the sloping margins of the pond. From the habitat dimensions given above, and allowing for areas subject to dense shade, about 25% of the pond perimeter has shallow water providing warmer temperatures in summer. However, hosts occur infrequently in these areas in daylight (when at increased risk of aerial predation), so only a small subset of the overall output of parasite eggs will be deposited here. With vertical walls surrounding most



of the pond, most eggs will settle in deeper water and predictions relating egg development to recorded temperatures should be realistic.

#### *Effects of temperature on infective stages*

The temperature intervals employed in the current study (10, 12, 15, 18, 20, 25°C) span the breakpoint in transmission attributable to temperature effects on egg development and survival. Jackson (1993) found that eggs incubated at 10°C for 2 months were arrested but retained high viability; when transferred to 20°C, their development resumed and hatching subsequently occurred after the period normally required at this higher temperature. However, the activity of hatched larvae appeared to be compromised after exposure of eggs to cold for >1 month and embryo viability was reduced after 3 months of arrest. The present trials showed that all eggs died when maintained for over 3 months at 10 and 12°C. This has the inevitable outcome that eggs produced by *P. xenopodis* make no contribution to transmission for the major part of the year. In this and a previous study by Jackson *et al.* (2001), 15°C represents the minimum temperature for embryogenesis.

As noted in accounts of other monogeneans (e.g. Kearns, 1986; Whittington *et al.* 2000) and of *P. xenopodis* in particular (Jackson *et al.* 2001; Jackson and Tinsley, 2007), eggs produced during one 24h period and developing under uniform conditions hatch over many days. This may have adaptive significance as a risk-spreading strategy to exploit mobile hosts that are only intermittently encountered. The temporal distribution of larval emergence may also achieve greater heterogeneity of parasite genotypes in established infrapopulations, with relevance to out-breeding. However, where there is an abrupt temperature-driven cut-off in hatching, as in Wales, this trait – otherwise presumably advantageous in transmission – may be responsible for considerable loss of reproductive output (see below and Fig. 4).

It might be predicted that the environmental conditions in Wales should contribute to strong selective pressure, operating during the past 40 years, for faster egg development and hatching. Jackson *et al.* (2001) recorded variations in hatching schedule between samples from different geographical areas in Africa. Thus, overall hatching windows at 15°C for 2 isolates of *P. xenopodis* (from the Cape and Swaziland respectively) were 49-79 and 56-88 days post-collection, compared with present data from the Welsh population of 57-77 days. These ranges might indicate a reduction in the hatching window in Wales (from about 30 to 20 days), but it would require more intensive studies to detect whether there has been a shift in temperature tolerance and/ or enhanced development at relatively low temperatures. At the threshold for development, 15°C, viability was only 25% in samples from the Welsh population whereas Jackson *et al.* (2001) recorded viability >95% at this temperature in samples from S. Africa and Swaziland. If significant, this change would be in the opposite direction to that predicted; however, since there is a steep decline in viability near to 15°C (and zero viability with a further 3°C decrease), small variations in experimental conditions and in parasite effects could produce these differences between studies.

#### *Interpretation of life cycle events in the natural habitat in Wales*

From the combined evidence of this and previously published studies (including Jackson and Tinsley, 1998a and reviews in Tinsley, 2004, 2005), it is possible to

predict a general schedule for the production of infective stages affecting the *X. laevis* population in Wales.

*Constraints on egg production.* Jackson and Tinsley (1998a) measured the effect of temperature on egg production rate in *P. xenopodis* by transferring two groups of infected *X. laevis* along a thermal gradient from 20 to 6°C (with delays for acclimation at each level). In these hosts, mixed with respect to age, gender, experience of infection and hence immune state, mean per group output ranged from about 12 e/w/d at 20°C to <1 e/w/d at 6°C. On the basis of these data, egg production in the Welsh population may just exceed 10 e/w/d at the highest summer temperatures. However, with average temperature between 1 June and 1 September 16.2°C in 2007 and 15.5°C in 2008, the mean maximum output is probably more usually about 9 e/w/d during June, July and August (the rate at 16°C recorded by Jackson and Tinsley (1998a)). Significantly, egg production can continue even at temperatures equivalent to those in mid-winter in Wales. At 10°C, the *per capita* daily rate is 1-2, but for more than half of each year output is probably <1 e/w/d (see Jackson and Tinsley, 1998a).

*Timing of egg development.* Data in Fig. 3 show that eggs require 5-7 weeks to hatch at 18°C, but this temperature was reached in the 2 summers shown in Fig. 1 on only 2-6 days. So, minimum hatch time at the field site is likely to be closer to the 8-11 week timescale recorded at 15°C. Water temperatures in 2008 did not exceed 15°C until early May, frequently dropped below this level during early summer, and then fell consistently below 15°C in early September.

The beginning of the period when egg production can contribute to transmission includes output during early spring (at 10-12°C) when eggs remain undeveloped but survive until rising temperatures in early May permit embryogenesis. Eggs produced during this time will add to a pool of larvae that will begin hatching after about 8 weeks of temperatures above 15°C. Egg output rates are low at spring temperatures but the accumulation of eggs, beginning development more or less simultaneously, could contribute to an enlarged infective larval population once hatching begins. In the thermal environment at the study site, the extent of this contribution is likely to be limited. Jackson *et al.* (2001) showed that brief exposure to low temperature shock (5°C for 18h followed by incubation at 25°C) had a small but significant effect on embryo viability. In Wales, sudden decreases over several days in early Spring (declining to <6°C in March in both 2007 and 2008, see Fig. 1) may depress viability and infectivity: temperatures did not reach 10°C consistently (excluding erratic spikes) until late March in 2007 and late April in 2008. So, the contribution to transmission made by eggs arrested at low temperatures and then recovering to complete development is likely to be restricted to only a few week's output (at only about 2 e/w/d) before the onset of temperatures  $\geq 15^\circ\text{C}$ .

*Timing of host invasion.* The start of the period of transmission can be predicted from the requirement for 8 weeks of water temperatures  $\geq 15^\circ\text{C}$ . In both 2007 and 2008, the onset of favourable conditions in early May was interrupted by a return of temperatures declining to 12°C, repeated briefly in June, that would have retarded embryo development rates. There may have been some compensation from spikes  $\geq 18^\circ\text{C}$  (especially in 2007) so a reasonable estimate for the start of hatching would be late June (2007) to early July (2008).

The end of the period of transmission in autumn is determined by the point at which eggs fail to hatch because their development is not completed before arrest at declining temperatures. Present studies did not assess the extent to which partly-developed eggs could complete development and hatch at temperatures immediately below 15°C, but embryogenesis is arrested completely at 12°C. Fig. 1 shows that, in both recorded years, there was a steep decline from 15 to 10°C in the second (2008) or third (2007) weeks of September and fluctuations did not rise above 13°C after 1<sup>st</sup> October (both years). Even if brief ‘spikes’ around 13-15°C in late September could permit minor development (as in 2007) and even if emergence might continue at 1 or 2°C below the threshold determined in this study, the time extension during rapidly falling temperatures would be very short (i.e. only a small additional contribution to infection after temperatures have declined <15°C). Therefore, in both 2007 and 2008, host infection is likely to have stopped by late September.

*Constraints on transmission.* With an end to hatching in late September and a minimum developmental period of 8 weeks, eggs produced after the start of August have insufficient time to complete development. In practice, this cut-off may be about 1 week later because eggs may experience fluctuations between 14 and 17°C during their initial development in August (Fig. 1). However, the limit on reproduction is more severe than this. The extended period of the ‘hatching window’ leads to failure to hatch for an increasing proportion of eggs produced in the 3 weeks preceding the final cut-off. To illustrate the principle involved, Fig. 4 is based on a timescale of 8 weeks for development and 3 weeks for hatching at 15°C (in reality, these periods may be a few days less because of the slightly higher temperatures experienced in part of July/ August). Assuming a final end-point of 7 August, only the output up to about 14 July will result in complete hatching of each day’s production. For eggs produced 1 week later (around 21 July), about 20% of daily output will not hatch; then one week after this (28 July), about 77% of eggs produced will fail to hatch; finally, during the 3<sup>rd</sup> week, even the small remainder will be eliminated by the ‘shutdown’ at declining temperatures. This represents a considerable loss of reproductive investment made at a time when water temperatures (and parasite life cycle rates) are near to their highest in the year. The rapid decline, hatching reduced from 80% to 23% between the 1<sup>st</sup> and 2<sup>nd</sup> weeks, is determined by the shape of the frequency distribution in Fig. 4.

From these calculations, the overall period during which egg production actually contributed to transmission in 2007 would have extended from late March to early August, a maximum of about 19 weeks, and in 2008 from late April to early August, about 15 weeks. However, the contributions at both ends of this period are reduced. In the first few weeks, output would have been very low, with reduced viability/ infectivity and a hatching schedule little more advanced than that of eggs newly produced in May. During the final 3 weeks, a declining proportion of eggs would have hatched. The period of output free of these restrictions and contributing to transmission is actually May, June and half of July, about 10 weeks. All eggs produced between early August and late March/ late April will die without hatching, over 7 months of reproductive output in 2007 and over 8 months in 2008.

There may be a limited increase to this schedule of transmission involving eggs laid in warmer shallow water where development may be accelerated (perhaps by 1-2 weeks) and the period of infection extended. However, the structure of the pond habitat and

predator avoidance behaviour by the host limit the output of parasite eggs in these more favourable micro-environments.

The temperature records for 2007 and 2008 illustrate the strong effect of year-to-year environmental variations on transmission involving relatively short-lived infective larvae. The duration of water temperatures  $\geq 15^{\circ}\text{C}$  was over 3 weeks longer in 2007 than in 2008 (attributable principally to the early boost to the seasonal thermal cycle from exceptionally warm conditions in April, see above). Because this temperature represents a critical threshold for egg development of *P. xenopodis*, variations around this cut-off have a comparatively major effect on transmission: potentially increasing the period of invasion by about 30%. In addition to their extended duration, the warmer temperatures in 2007 (represented by the greater area under the curve of temperatures  $>15^{\circ}\text{C}$ , see above and Fig. 1) will have maintained increased rates of egg output and egg development in comparison with 2008, contributing further to enhanced invasion rates. For helminths with development thresholds near to this critical  $15^{\circ}\text{C}$  boundary, even small increases in 'climate warming' may have a significant effect on parasite infection.

In summary, the life cycle metrics of *P. xenopodis* are strongly limited in an environment dominated by low temperatures. Parasite egg output is continuous throughout the year, but eggs produced during 7–8 months each year are destined not to hatch. Temperatures allowing infective stages to develop extend from late April/early May to mid September but host invasion begins only in late June/early July. The present data pinpoint the period of parasite egg production that actually contributes to transmission: 15–19 weeks per year in the 2 study years. However, exclusion of periods when egg output is very low (in April) and when hatch rate is declining (late July), indicates a period of effective reproductive output of only about 10 weeks per year (early May to mid July). As a consequence, when egg output rates are near their maximum, in early-mid August, the window for completion of egg development has already closed. The overall period of host invasion is similar, about 10 weeks/ year, but this is staggered by about 8 weeks with respect to corresponding egg output. Invasion is probably precluded entirely for about 9 months each year, between late September and late June/early July. The halt in autumn is attributable to the failure of eggs to complete development at declining temperatures; the continued inhibition until the following early summer reflects the time-lag after favourable temperatures return in spring before infective larvae complete development.

The establishment of an African parasite in a cool north temperate environment presents an exceptional opportunity for understanding limiting factors in transmission biology. This study has quantified the schedules for development and host invasion in the natural habitat. Clearly, the persistence of this infection in Wales for at least 40 years indicates that the very restricted input of infective stages can maintain the parasite population. The effects of temperature on these stages subsequently within the host are of major importance for understanding the survival of this introduced parasite: these are considered in an accompanying account (Tinsley *et al.* 2010).

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## Captions to Figures

- Fig. 1. Water temperatures at the study site pond, Wales for Dec 2006 – Dec 2008. Horizontal bars indicate the periods when temperatures exceeded 15°C (▨), 12°C (▩) or were below 10°C (▧). Durations were selected on the basis of abrupt major changes in temperature (in both directions), ignoring subsequent short-term fluctuations.
- Fig. 2. Effect of incubation temperature (each  $\pm 0.05^\circ\text{C}$ ) on viability of embryos of *P. xenopodis* (bars show means  $\pm$ S.E., samples sizes below). Pair-wise differences between temperatures are statistically significant except 10-12 and 18-20°C.
- Fig. 3. Frequency distributions of hatching times of eggs of *P. xenopodis* incubated at 15°C (□), 18°C (▣), 20°C (▤) and 25°C (▥) (see text for sample sizes). The difference between the overlapping ‘hatching windows’ at 18 and 20°C is statistically significant.
- Fig. 4. The effect of the egg ‘hatching window’ of *P. xenopodis* on emergence of infective stages at the end of the transmission season. Timing is based on development at 15°C (close to the minimum for egg viability and the mean temperature in the natural habitat in Wales for May – September), and shows the final 3 weeks of the 11 week schedule of development/ hatching at this temperature. The upper part shows proportions of larvae hatching per day (bars) and per week (curved lines), beginning on day 56 day after production and completed on day 77. The lower part depicts the fate of a single day’s production of eggs in the period preceding inhibition of hatching by declining temperatures. The curves indicate the proportions of the larval population that are lost when there is a 1, 2 or 3 week reduction in the hatching period before termination (because of later production of eggs or earlier onset of low temperatures that close the ‘hatching window’).