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Climate changes influence free-living stages of soil-transmitted parasites of European rabbits

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Abstract

Climate warming has been suggested to augment the risk of infectious disease outbreaks by extending the seasonal window for parasite growth and by increasing the rate of transmission. Understanding how this occurs in parasite-host systems is important for appreciating long-term and seasonal changes in host exposure to infection and to reduce species extinction caused by diseases. We investigated how free-living stages of two soil-transmitted helminths of the European rabbit (*Oryctolagus cuniculus*) responded to experimental changes in temperature by performing laboratory experiments with environmental chambers and field manipulations using open-top-chambers. This study was motivated by our previous observations that air temperature has increased over the last 30 years in our field site and that during this period intensity of infection of *Graphidium strigosum* but not *Trichostrongylus retortaeformis* was positively associated with this temperature increase. Laboratory and field experiments showed that both parasites accelerated egg development and increased hatching rate and larval survival in response to accumulating thermal energy. Both parasites behaved similarly when exposed to diverse temperature regimes, decadal trends, and monthly fluctuations, however, *T. retortaeformis* was more successful than *G. strigosum* by showing higher rates of egg hatching and larval survival. Across the months, the first day of hatching occurred earlier in warmer conditions suggesting that climate warming can lengthen the period of parasite growth and host exposure to infective stages. Also, *T. retortaeformis* hatched earlier than *G. strigosum*. These findings showed that seasonal changes in intensity, frequency, and duration of daily temperature are important causes of variability in egg hatching and larva survival. Overall, this study emphasizes the important role of climate warming and seasonality on the dynamics of free-living stages in soil-transmitted helminths and their contribution to enhance host exposure to parasitic infections. Yet, the ability to infect might ultimately depend on how hosts interact with parasites.

Keywords: decadal trends, European rabbit, gastro-intestinal helminths, seasonality, temperature regimes, thermal accumulation

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Introduction

Seasonal phenology, individual physiology, and population dynamics are intimately tied to the natural climatic regimes that animals and plants experience in their geographic distribution (Davis, 1981; Morin, 1984, 1999; Kochmer & Handel, 1986). Global climate changes and local weather alterations have been suggested to affect processes both at the individual and population level and predicted to influence trophic interactions and community properties such as species composition and structure (Morin, 1999; Hughes, 2000; Wuethrich, 2000; Walther *et al*., 2001, 2002; Dillon *et al*., 2010). Current observations from natural ecosystems have highlighted the general negative consequences of climate-related change, including an increasing number of reports on habitat disruption, range shift, and species extinction (Thomas *et al*., 2004; Malcom *et al*., 2006; Pereira *et al*., 2010; Altizer *et al*., 2011; Beaumont *et al*., 2011). Global warming has also been associated with augmented risk of disease outbreaks (Dobson & Carper, 1992; Harvell *et al*., 2002; Rohr *et al*., 2011).

Among infectious disease ecologists, the general consensus is that climate-driven intensification of host-parasite interactions and lengthening of the parasite reproductive season can create opportunities for the invasion of a novel infectious agent or facilitate the transmission of an already circulating infection, ultimately leading to higher prevalence and expansion of diseases into naive host populations (Harvell *et al*., 2002; Dobson *et al*., 2006; Johnson & Thieltges, 2010; Keesing *et al*., 2010; Rohr *et al*., 2011). However, whether climate warming will effectively promote the emergence and/or intensify the circulation of infectious diseases remains controversial (Hay *et al*., 2002; Rohr *et al*., 2008; Dobson, 2009; Harvell *et al*., 2009; Lafferty, 2009; Pascual & Bouma, 2009; Randolph, 2009). Part of this debate is that interactions between climate and parasites often exhibit nonlinear relationships and transmission is strongly

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influenced by the behavioral and physiological components of the parasite, the host and the intermediate hosts or vectors (McMichael *et al.*, 2006; Halstead, 2008). For example, although climate warming can expand the geographic distribution of vector borne infections, by increasing vector biting rate and parasite replication, high temperatures can also have a negative effect on vector survival (Paajimans *et al.*, 2009, 2010; Lambrechts *et al.*, 2011). Similarly, socioeconomic and ecological forces driving human, livestock and wildlife movements can weaken the net effect of climate related changes to the spatial and temporal patterns of infections (Kuhn *et al.*, 2003; Lafferty, 2009; Morgan & Wall, 2009; Altizer *et al.*, 2011). One clear conclusion stemming from these arguments is that there is a need for more empirical work as well as long-term data from a larger suite of parasite-host systems to gain a more rigorous understanding of the complexities influencing the interaction between climate and infectious agents and to better frame large-scale and long-term predictive models of infectious disease dynamics.

Parasitic helminths play a critical role in ecosystem functioning and food web structure and continue to represent a health and economic problem of global significance (Combes, 1996; Bundy & de Silva, 1998; Stoll, 1999; de Silva *et al.*, 2003; Lafferty *et al.*, 2006, 2008). Over the recent decades there has been growing evidence that prevalence and intensity of helminths has increased in wildlife and livestock from temperate and subarctic latitudes and this has been frequently explained as result of a general warming of these areas (Kutz *et al.*, 2005; Hudson *et al.*, 2006; van Dijk *et al.*, 2008; Kenyon *et al.*, 2009). Helminths often produce free-living stages whose development and survival is strongly influenced by the climatic conditions of their microhabitat and by the extent of cold and heat stressors they can tolerate (Croll, 1966; Read, 1972; Bush, 2001; Wharton, 2002). Experimental studies on soil-transmitted helminths (e.g. *Haemonchus contortus*, *Hetereakis gallinarum*, *Nematodirus battus*, *Ostertagia ostertagi*, *Trichostrongylus* spp) have found that egg hatching and larval development were accelerated in warmer temperatures, but excessive high values had a negative effect on their survival (Crofton, 1948b; Ciordia & Bizzell, 1963; Thomas, 1974; Stromberg, 1997; Saunders *et al.*, 2002; van Dijk & Morgan, 2008). Eggs exposed to stochastic fluctuations in temperature also developed faster when compared to constant or cyclic temperature regimes (Saunders *et al.*, 2001, 2002). Together, these studies support the hypothesis that the intensity, duration, and frequency of thermal cues are crucial for the development and survival of helminth free-living stages. A few recent studies have explicitly addressed the role of climate warming on parasite dynamics (van Dijk & Morgan, 2008; Koprivnikar & Poulin, 2009; Studer *et al.*, 2010; Morley, 2011; Paull & Johnson, 2011) yet, how parasites respond to daily, seasonal, and long-term climate changes remains poorly understood.

In this study, we examined the effect of climate changes on the dynamics of free-living stages of two soil-transmitted, poikilothermic gastrointestinal helminths in an herbivore system. We previously found a positive relationship between air temperature (°C) and intensity of infection for *Graphidium strigosum*, but not *Trichostrongylus retortaeformis* in a population of European rabbits (*Oryctolagus cuniculus*) monitored monthly from 1977 to 2002 in Scotland (UK) (Hudson *et al.*, 2006; Harvell *et al.*, 2009). Between 1980 and 2002 mean air temperature increased by 1 °C in the study site and an updated analysis of the temperature data through 2009 has shown that this trend has been consistent (Fig. 1) (Hudson *et al.*, 2006; Harvell *et al.*, 2009). Both helminths exhibit strong seasonal transmission but contrasting dynamics of infection (Cattadori *et al.*, 2005, 2008; Cornell *et al.*, 2008). Rabbits develop an immune response that can reduce or remove *T. retortaeformis*, but not fully protect against re-infections; in contrast, *G. strigosum* accumulates with host age with no clear evidence of parasite mortality, despite a robust species-specific immune response (Cattadori *et al.*, 2005, 2008; Murphy *et al.*, 2011). On the basis of these observations, we predicted that the free-living stages of the two helminths would exhibit similar responses to climatic constraints and that the contrasting long-term temperature-intensity relationships observed in the field have been caused by the dis-

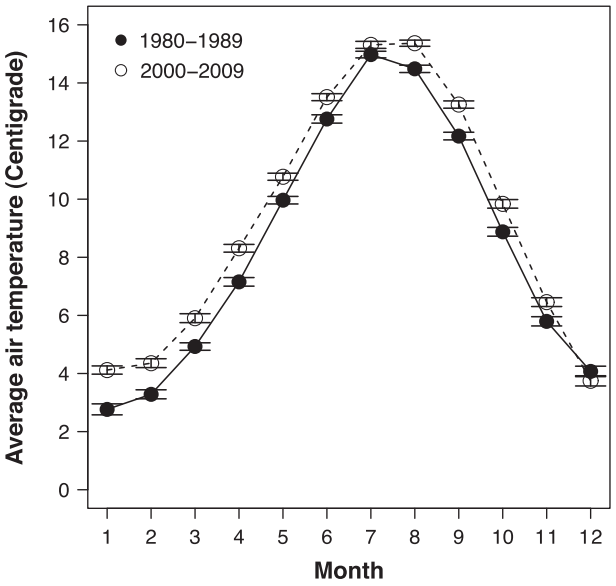

Fig. 1 Monthly air temperature in the cold decade, 1980–1989, and warm decade, 2000–2009, from our long-term field study in Scotland. SE bars are reported.

tinct interactions with the host. This study focused on the free-living stages and asked how daily, seasonal, and long-term climatic changes influenced egg hatching rate and larval survival of the two helminths, using a combination of laboratory and field experiments. These parasites are closely related to helminths of sheep and cattle and findings from this study will contribute to better understanding the potential role of climate change on helminth infections of livestock and more broadly, natural animal systems with similar parasite species.

Materials and methods

The study system

Parasites' life history. *Graphidium strigosum*, and *T. retortaeformis* are gastrointestinal helminths of the European rabbit and other lagomorphs (Audebert & Durette-Desset, 2007). Host infection occurs by direct ingestion of contaminated herbage with infective larvae; *G. strigosum* colonizes the stomach while *T. retortaeformis* inhabits the small intestine, reproduction is sexual and eggs are shed into the environment with the host feces (Audebert *et al*., 2002; Massoni *et al*., 2011). The time between infection and the appearance of eggs (prepatent period) for *G. strigosum* is 42–44 days (Massoni *et al*., 2011), and 12–13 days for *T. retortaeformis* (Audebert *et al*., 2002). Infective larvae can persist on the herbage for a year or more (Michel, 1974). *T. retortaeformis* eggs dropped on the pasture in autumn, survived cold winters, and hatched in spring, whereas eggs spread during the winter died quickly (Crofton, 1948b). Studies on larval behavior found that the majority of *T. retortaeformis* infective larvae migrated daily up and down the grass blades in relation to temperature and humidity, protection was sought in herbage or soil during the warmer summer months and in the detritus mat layer during the spring and autumn periods (Crofton, 1948a). Laboratory experiments on *T. retortaeformis* have shown that increasing air temperature accelerated eggs hatching, but reduced larval survival: the earliest hatching was 19 h at 30–35 °C while the latest at 9–12.5 days at 5 °C, no hatching was recorded at 40 °C; there was no survival of larvae above 35 °C (Prasad, 1959; Gupta, 1961). We are not aware of studies on *G. strigosum* free-living stage behavior in relation to climatic cues, although it has been suggested that eggs hatch within 8–10 h at 20 °C and that free-living larvae can be adversely affected by desiccation (Wetzel & Enigk, 1937; Enigk, 1938).

Field study site. Our hypotheses and previous observations on the helminth-climate-rabbit system build on observations from long-term data collected from a managed agro-ecosystem in Perthshire, Scotland (Cattadori *et al*., 2005, 2008; Hudson *et al*., 2006; Harvell *et al*., 2009). The climate of this region is temperate with strong seasonality in temperature and a less obvious trend for rainfall: the warmest months are July and August, and the coldest December, January, and February (Data S1: Fig. S1, S2). The field manipulation (see *Climatic manipulations in the field*) was performed in an adjacent area (Latitude 56° 24′ 41.22″ N, Longitude 3° 16′ 47.59″ W) with similar habitat and land use characteristics.

Climatic simulations in the laboratory

Climatic data. To examine the effect of different climatic scenarios on *G. strigosum* and *T. retortaeformis* free-living stages, parasite eggs were exposed to a number of temperature regimes in climatic chambers. Three settings were tested: Constant, Cycle, and Stochastic using three programmable incubators selected at random among the trials (Model 2005; VWR International, Radnor, PA, USA, two fitted with microprocessor Series 982; Watlow Controls, Pittsburgh, PA, USA). The chambers were not designed to control for humidity, however, eggs were spread on 1% agarose in Petri dishes that maintained close to 100% humidity condition throughout the trials. We selected this medium, rather than using a simple water solution, to allow enough moisture while providing a more natural substrate for the larvae to move around. Temperature-humidity data recorders (i-button®; Maxim Integrated Products, San Jose, CA, USA) were set up in each chamber for the duration of the experiments to confirm the consistency of the temperature data (°C), programmed *a priori*, and to provide accurate measurements of temperature and humidity changes every 10 min. These temperature data were then used in the analysis. The temperature regimes were based on the air temperature recorded daily at the meteorological station of The James Hutton Institute (Invergowrie, Scotland), located approximately 10 miles from our rabbit population, where long-term trends between temperature and intensity of infections were originally observed (details in Data S1; Harvell *et al*., 2009).

Five Constant temperatures were simulated: 5, 10, 15, 20, and 25 °C, which represent the range of mean air temperature that free-living stages are exposed to during the spring-to-autumn months in our long-term study site in Scotland, and cover the majority of the temperate range the two helminths can tolerate (Data S1: Fig. S2; Anderson, 2000). The Cycle and Stochastic regimes followed a 12 : 12 h daily cycle and were based on the minimum and maximum temperatures recorded in the months of March, May, July, September, and November between 1980 and 2009. These months are representative of spring, summer, and fall seasons when exposure to the worm contaminated herbage is at the highest compared to the winter months and span across the rabbit breeding period (April through August). For the Cycle regime, average monthly minimum and maximum temperatures were used and temperature ramped in gradual steps toward these extremes over a 12 h period. The Stochastic regimes mimicked the Cycle pattern, but daily minimum and maximum temperatures were randomly selected (RAND, function in Excel; Microsoft Corporation, Redmond, WA, USA) for every simulated month as follows: the daily minimum temperatures were randomly chosen between the average minimum temperature and the lowest minimum temperature range, whereas the daily maximum temperatures were randomly chosen between the average maximum temperature and the highest maximum temperature range. Again, chambers were programmed to reach these daily max–min

extremes in gradual steps over the 12 : 12 h cycle. To capture the long-term climate warming recorded in our study site from 1980 to 2009 (Fig. 1), two sets of data were selected for the Cycle and Stochastic regimes: monthly temperatures from decade-1 (years: 1980–1989), as representative of 'low temperature' years, and monthly temperatures from decade-2 (years: 2000–2009), as indicative of 'high temperature' years (Fig. 1 & Data S2: Table S1; Fig. S3). Specifically, for both the Cycle and Stochastic regimes, temperature data for March, May, July, September, and November were estimated in each decade. By examining the temperature regimes from different months in different decades we aimed to capture seasonal variations within and between decades and to identify crucial months that may have exerted the strongest climatic effect on the hatching of eggs, and whether these key months have changed from the cold to the warm decade. This approach allowed us to provide a link with the field climatic manipulations (details below) and to assist in the understanding of the seasonal and long-term dynamics of the two helminth infections in the natural rabbit population. Overall, a total of 25 temperature regimes were tested (see Data S2: Table S2 for summary).

Parasite data. For every climatic simulation, eggs were extracted from fresh feces collected from rabbits single infected with the two helminths within 12 h of defecation and for each parasite the feces were mixed to provide a homogenous pool. Briefly, feces were diluted in water (15 ml g^{-1}), mechanically blended, filtered through a 212 μm sieve, and the supernatant poured into 50 ml tubes that were centrifuged for 10 min at 1500 g and 4 °C. The supernatant was discarded, approximately 0.02 g of kaolin powder added to the pellet in each tube, and a saturated NaCl solution was then added to have 14 ml solution, which was then centrifuged for 10 min at 2500 rpm and 4 °C. The supernatants were poured into a 36 μm sieve and left under a running cold tap water for 30 min to wash the kaolin and NaCl off the eggs. Finally, eggs were pooled into 20 ml of water, and the total number estimated with MacMaster counting chamber slides (Gordon & Whitlock, 1939). The entire protocol was repeated for each helminth species using separate sieves and tubes to avoid cross contamination.

A fixed amount of eggs was placed into 60 mm Petri dishes filled with 10 ml of 1% agarose (range of eggs/dish used 32–392 based on each species' availability). Six dishes for each parasite species were randomly collected from the incubators at each sampling point and the number of larvae and eggs in every dish counted. The position of the remaining dishes was also shuffled each time to avoid any possible effects of temperature variations within chambers. All Constant temperature regimes were sampled on days 0, 1, 2, 3, 6, 7, 8, and 9, except for the 5 °C treatment, which was sampled on days 0, 3, 6, 8, 9, 10, 12, 14, and 16, to take into account the slower maturation of eggs. Cycle regimes were sampled on days 0, 1, 2, 3, 4, 6, 7, 8, and 9, except for the colder months of March and November that were sampled on days 0, 3, 6, 9, 10, 11, 12, 14, 16, and days 0, 3, 5, 7, 8, 9, 10, 12, and 13, respectively. Similarly, Stochastic regimes were sampled on days 0, 1, 2, 3, 6, 7, 8, and 9, except for March and November, which were sampled on Days 0, 3, 6, 7, 8, 9, 10, 12, 13, and Days 0, 3, 5, 6, 7, 8, 9, 11, and 13, respectively. To account for the disparity in sampling time between trials, analyses were based on data collected in the first 9 days of each trial (see details below). Also, to allow statistical comparison and a balanced design, the missing sampling points that preceded the day when at least one egg hatched were included as zero eggs hatched so that data from an equal number of sampling points, i.e. 9, were available for each experiment.

Climatic manipulations in the field

Climatic data. To determine the response of free-living stages to experimental alterations of natural daily and seasonal climatic fluctuations, open-top chambers (OTC, Hollister & Webber, 2000) were used to manipulate air temperature and humidity in the field from April to October 2010. Fiberglass truncated cone chambers (84.6 cm bottom diameter × 50 cm top diameter × 30 cm height, Solar Components Corporation, Manchester, NH, USA) were built following the International Tundra Experiment (ITEX) manual (see Fig. 3 in Marion, 1996). The shape and size of the OTCs were designed to increase the internal temperature between 1–2 °C, within the range of temperate changes recorded in our study area over the last 30 years (Hudson *et al.*, 2006). A grid of five OTC and five open herbage controls, 1.7 m apart, was set up in an exclosure that prevented rabbit, livestock, and deer disturbance. A temperature-humidity data recorder (i-button®; Maxim Integrated Products) was placed in the middle of each OTC and control 20 cm from the ground, and the top shielded with a white plastic roof from direct solar radiation; data (temperature in °C) were recorded every 10 min for the duration of each experiment. A comparison with the climatic records from The James Hutton Institute meteorological station (~12 miles apart) confirmed the strong correlation between air temperature data from the two datasets (Data S3: Fig. S4).

Parasite data. For every OTC and control, two 90 × 20 cm pieces of rye-fescue grass mix lawn-turf (2.5–3.0 cm tall), one for each helminth species, were set into bare soil 1 week prior to the experiment, with a 5 cm gap around and between the two turf pieces filled with potting soil. Each piece was divided into 10 × 10 cm squares and each square seeded with an equal mass of rabbit feces collected from laboratory rabbits single infected with either *G. strigosum* or *T. retortaeformis*. Rabbit feces were collected daily and stored at 4 °C during the week leading the beginning of each experiment (range of monthly mass of feces available: *G. strigosum* 1125–2870 g and *T. retortaeformis* 280–2430 g). For every parasite, the weekly feces were mixed, the average number of parasite eggs per gram of feces (EPG) from three random sub-samples estimated at time zero (Gordon & Whitlock, 1939) and finally, an equal amount of feces and parasite eggs was placed on every 10 × 10 cm turf-grass square on day 0 (range of monthly EPG for *G. strigosum:* 952–9200 and *T. retortaeformis:* 894–12 690). For each helminth, one square of turf-grass was removed from every OTC and control every other day starting on day 5. Experiments were repeated every 4 weeks, and the duration of each experiment was 15 days in April and extended to 17 days in May through July and 19 days in August through October. No experiments were performed in the winter months.

For each turf-grass square, the remaining feces were collected and the number of eggs estimated. Third stage larvae were recovered using an adaptation to the Baermann technique (Baerman, 1917). Briefly, grass squares were inverted on Miracloth filter paper in warm tap water in plastic funnels overnight. Percolated larvae were then collected and two to three drops of pharmacy grade iodine added to 10 ml of water to kill and stain free-living helminths, but not the sheathed parasitic infective larvae, which remained alive and could be easily counted under a stereomicroscope. All laboratory procedures on rabbit single infections with the two helminths were conducted at the University of Glasgow with the kind support of Prof. M. Stear and performed according to UK regulations with the approval of The Home Office (UK).

A few assumptions had to be made for the analysis of these data. First, that 100% of eggs were viable and would have hatched and second, that the mortality of free-living stages (eggs, larval stages 1, 2, and 3) due to biotic factors (e.g. predation) was constant and comparable within and between experiments.

Statistical analysis

To examine the effect of different temperature regimes, long-term warming and seasonality on the hatching rate of eggs of both helminths, Linear Mixed Effect Models (LME-REML, *lme* package NLME in R, Pinheiro & Bates, 2000) were implemented to the laboratory dataset. Egg hatching rate, as the response variable, was examined in relation to the following fixed explanatory variables: temperature regimes (Constant, Cycle and Stochastic), time (sampling day), decade (cold and warm), month (March, May, July, September, and November) and thermal energy accumulation (cumulative degree day, which is a relative measure of the thermal energy necessary for organism development, Data S4 for details). A hierarchy of models was examined based on diverse combinations of independent variables. The variability between climatic chambers was included as a random effect, described as standard deviation of the model intercept. Preliminary analysis also confirmed the high consistency among the Petri dish counts and thus they were removed from the random component of the model. Decade and month were also included as random factors depending on the model examined. Data exploration started from a full additive model and the contribution of two-way and three-way interaction between fixed effects was also examined. The proportion of eggs hatched was arcsin-transformed, thermal energy accumulation was log $(x + 1)$ transformed and temporal variables (regime, decade and month) were represented as factors. The minimum parsimonious models (significance threshold: $P = 0.05$) were presented as final results.

Linear mixed effect models were also used to examine whether the phenology of the first day of hatching was affected by the climatic regime and seasonality. Only the sampling points between the start of the experiment and the day when eggs in all Petri dishes had hatched were considered. The variability between the two climatic chambers was included as a random effect and mean hatching day (based on the number of positive Petri dishes) was log $(x + 1)$ transformed.

To examine how the manipulation of climatic variables affected the recovery of infective larvae in the field experiments, an adaptation of Generalized Linear Model with negative binomial error was applied (GLM, *glm.nb* package MASS in R, Venables & Ripley, 2002) to take into account the use of proportion data as a response variable. Based on the mathematical similarity that $\log(y/x) = \log(y) - \log(x)$, the count of infective stages, $\log(y + 1)$ transformed, was used as the response variable and the number of eggs set up on day 0, $\log(x + 1)$ transformed and representing the denominator of the proportion, was included as a fixed intercept to the model. This approach was necessary because very few eggs were recovered at each sampling point from feces remaining on turf-grass, making it difficult to accurately quantify egg abundance and temporal changes in larval mortality. Treatment (OTC vs. control), helminth species, experimental month,and their two-way interactions were included as independent explanatory variables. Again, the minimum parsimonious models (significance threshold: $P = 0.05$) were presented as final results.

Results

Climatic simulations in the laboratory

Across the three temperature regimes, more eggs of both helminths hatched in the Constant relative to the Cycle or Stochastic simulations (Fig. 2a; Table 1a). Thermal energy (degree-days) accumulated over experimental time and more heat accumulated in the Constant than the two cyclic regimes (Fig. 2d; Table 1b). The proportion of eggs hatching increased with the accumulation of thermal energy and more *T. retortaeformis* eggs hatched compared to *G. strigosum* in the three regimes (Table 1a, Data S5: Fig. S6, Table S3).

To examine the rate of egg hatching in more detail and under more realistic climatic conditions, analysis focused on the cyclic regimes only. More eggs of both species hatched in the Stochastic than the Cycle trials, also more eggs hatched in the warmer relative to the colder decade (Fig. 2b; Table 2a). *T. retortaeformis* showed higher hatching than *G. strigosum*, with hatching increasing as thermal energy accumulated (Fig. 2b; Table 2a). Accumulation of thermal energy was higher in Stochastic relative to Cycle regimes and in the warmer relative to the cooler decade (Fig. 2e; Table 2b).

We next investigated the role of seasonal changes in temperature on the rate of hatching. For both parasite species, hatching was at the highest in July and at the lowest in March and November in the Cycle and Stochastic simulations (Fig. 2c; Table 3a). Hatching was lower in Stochastic relative to Cycle regimes in summer and vice versa in spring and autumn (Fig. 2c; Table 3a). In both cyclic regimes, thermal energy exhibited a consistent seasonal pattern with the highest energy accumulated in July and the lowest in March and November

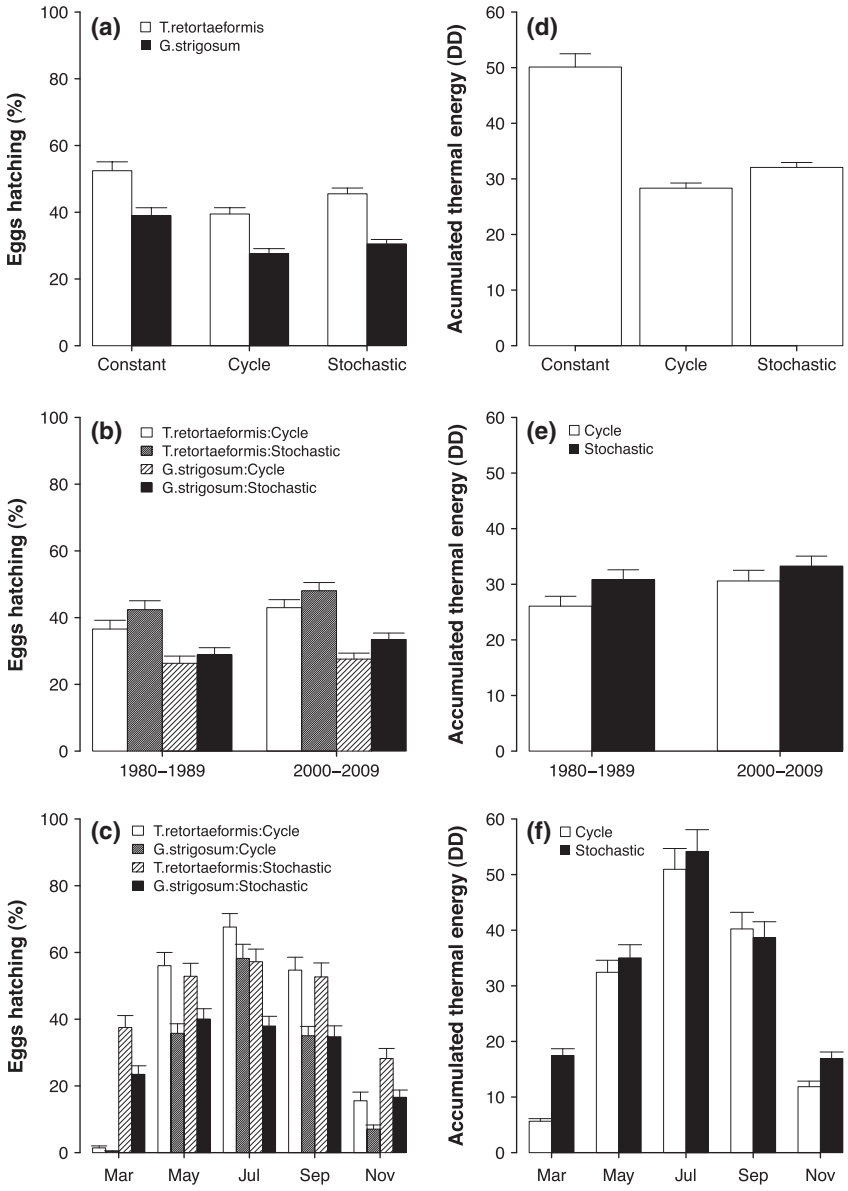

Fig. 2 Percentage of parasite eggs that hatched by: temperature regimes (a) decades (cool decade: 1980–1989 and warm decade: 2000–2009) in cyclic regimes (b) and months in cyclic regimes (c). Accumulated thermal energy by: temperature regimes (d) decades in cyclic regimes (e) and months in cyclic regimes (f). SE bars are reported.

(Table 3b). However, more thermal energy accumulated monthly in the Stochastic compared to the Cycle regime (Fig. 2f; Table 3b).

Overall, these findings highlighted analogous patterns, but different rate of responses between the two helminths when exposed to similar climatic changes. To identify species-specific climatic-related trends, an investigation was performed independently for each parasite species. For both helminths the pattern of hatching by regime and with thermal energy was consistent with previous observations (Fig. 3a–e, Data S5: Table S4, S5). Among the Cycle regimes and for both helminths hatching showed a strong seasonal trend, but no significant differences between decades (Table 4a, 4b; Fig. 3f–j). In the Stochastic regimes, in addition to the clear seasonal trend, more *T. retortaeformis* eggs hatched in the second decade in the month of July and showed a tendency to higher hatching for the month of May (Table 4c; Fig. 3k–o). For *G. strigosum*, in addition to the seasonal hatching, more eggs hatched in the warmer than the colder decade although no particular month appeared to drive this pattern (Table 4d; Fig. 3k–o).

Finally, the relationship between first day of hatching and temperature regimes was examined. Overall, eggs

Table 1 Linear mixed effect models between the proportion of eggs that hatched (a) or the accumulated thermal energy (DD) (b), as response variables, and temperature regimes (Constant, Cycle, and Stochastic), sampling day and helminth species (when necessary) as explanatory terms

	(a)			(b)		
	Coeff. ± SE	df	*P*	Coeff. ± SE	df	*P*
Intercept	0.03 ± 0.13	2445	0.792	1.25 ± 0.47	2464	0.008
Cycle	−0.16 ± 0.02	2445	<0.0001	−0.59 ± 0.05	2464	<0.0001
Stochastic	−0.16 ± 0.02	2445	<0.0001	−0.41 ± 0.05	2464	<0.0001
Sampling day (day)	0.11 ± 0.002	2445	<0.0001	0.36 ± 0.005	2464	<0.0001
Graphidium strigosum (G.s.)	−0.05 ± 0.02	2445	0.019			
*G.s.**day	−0.03 ± 0.003	2445	<0.0001			
Random factor: chamber	0.22			0.82		
AIC	947.9			5884.9		

Table 2 Linear mixed effects models between the proportion of eggs that hatched (a) or the accumulated thermal energy (DD) (b), as response variables, and regime (only Cycle and Stochastic), simulated decade, sampling day, DD, and parasite species, as explanatory terms

	(a)			(b)		
	Coeff. ± SE	df	*P*	Coeff. ± SE	df	*P*
Intercept	0.02 ± 0.02	1961	<0.0001	1.1 ± 0.2	1982	<0.0001
2000–2009	0.02 ± 0.01	1961	0.13	0.2 ± 0.03	1982	<0.0001
Stochastic	0.06 ± 0.01	1961	<0.0001	0.2 ± 0.03	1982	<0.0001
Graphidium strigosum (G.s.)	−0.08 ± 0.01	1961	<0.0001			
DD	0.02 ± 0.0004	1961	<0.0001			
2000–2009*DD	−0.001 ± 0.0004	1961	0.001			
Stochastic*DD	−0.003 ± 0.0004	1961	<0.0001			
*G.s.**DD	−0.003 ± 0.0003	1961	<0.0001			
2000–2009*Stochastic*DD	0.002 ± 0.0004	1961	<0.0001			
Sampling day				0.3 ± 0.004	1982	<0.0001
Random factor: month	0.05			0.54		
AIC	−394.7			3792.7		

hatched significantly earlier for *T. retortaeformis* (mean day ± SE: 4.08 ± 0.2) than *G. strigosum* (4.36 ± 0.2; coeff. ± SE = 0.10 ± 0.05, $P < 0.05$) and eggs hatched faster in Constant (mean day ± SE: 3.65 ± 0.3) than Stochastic (3.74 ± 0.14; Constant vs Stochastic: coeff. ± SE: 0.24 ± 0.07, $P < 0.001$) or Cycle trials (4.89 ± 0.26; Constant vs Cycle: coeff. ± SE: 0.34 ± 0.075, $P < 0.001$). Within the Constant regime, first-day of hatching decreased with increasing temperatures for both helminths (*T. retortaeformis*: coeff. ± SE = −0.00008 ± 0.00007, $P < 0.001$; *G. strigosum*: −0.00007 ± 0.00005, $P < 0.001$, Fig. 4a). Within the cyclic regimes, eggs hatched significantly earlier in the Stochastic than Cycle regime (coeff. ± SE: −0.08 ± 0.02, $P < 0.0001$), in the warmer second decade than during the cooler first decade (coeff. ± SE: −0.09 ± 0.02, $P = 0.0001$), and for *T. retortaeformis* than *G. strigosum* (coeff. ± SE: 0.07 ± 0.02, $P < 0.0001$). Analysis repeated for each species in the cyclic regimes confirmed previous observations, hatching occurred earlier in the Stochastic relative to the Cycle trials and in the warmer second decade; the earliest first day of hatching was recorded in the month of July (Fig. 4b, c; Table 5).

Climatic manipulations in the field

The manipulation of temperature in the field showed that more thermal energy accumulated in the OTCs relative to the controls over the experimental months (coeff. ± SE = −0.07 ± 0.01, $P < 0.001$; the negative sign is based on the labeling of the variables), this corresponded to an average (±SE) increase in temperature of 0.7 °C ± 0.10 (Fig. 5c, Data S6: Table S6). Relative humidity was not significantly different between treatments although exhibited a clear seasonal trend (Data S6: Table S7). More infective larvae were recovered from

Table 3 Linear mixed effects models between the proportion of eggs that hatched (a) or the accumulated thermal energy (DD) (b), as response variables, and regime (only Cycle and Stochastic), simulated month, and sampling day, DD, and species, as explanatory terms

	(a)			(b)		
	Coeff. ± SE	df	*P*	Coeff. ± SE	df	*P*
Intercept	−0.02 ± 0.02	1960	0.18	0.52 ± 0.1	1974	<0.0001
Stochastic	0.16 ± 0.02	1960	<0.0001	0.67 ± 0.06	1974	<0.0001
May	0.19 ± 0.02	1960	<0.0001	0.89 ± 0.08	1974	<0.0001
July	0.2 ± 0.02	1960	<0.0001	1.1 ± 0.08	1974	<0.0001
September	0.1 ± 0.02	1960	0.006	0.95 ± 0.08	1974	<0.0001
November	0.03 ± 0.02	1960	0.15	0.29 ± 0.01	1974	0.03
DD	0.01 ± 0.0002	1960	<0.0001			
Graphidium strigosum (G.s.)	−0.08 ± 0.01	1960	<0.0001			
*G.s.**DD	−0.003 ± 0.003	1960	<0.0001			
Stochastic*May	−0.2 ± 0.03	1960	<0.0001	−0.61 ± 0.08	1974	<0.0001
Stochastic*July	−0.4 ± 0.03	1960	<0.0001	−0.61 ± 0.09	1974	<0.0001
Stochastic*September	−0.14 ± 0.03	1960	<0.0001	−0.71 ± 0.09	1974	<0.0001
Stochastic*November	−0.1 ± 0.03	1960	0.0007	−0.37 ± 0.09	1974	<0.0001
Sampling day (day)				0.29 ± 0.01	1974	<0.0001
Day*May				0.07 ± 0.01	1974	<0.0001
Day*July				0.1 ± 0.01	1974	<0.0001
Day*September				0.08 ± 0.01	1974	<0.0001
Day*November				0.03 ± 0.01	1974	0.03
Random factor: decades	0.007			0.11		
AIC	−563.8			3708.2		

the OTCs compared to the controls (coeff. ± SE = −3.71 ± 1.19, $P < 0.001$), and the majority were *T. retortaeformis* (coeff. ± SE = −7.1 ± 1.67, $P < 0.001$). Analyses repeated for each species independently, showed that more infective *T. retortaeformis* larvae were recovered from OTC than controls, but with large variability between months, the highest numbers was recovered in July (Fig. 5a; Table 6a). For *G. strigosum*, there was no difference in the number of infective larvae collected between treatments across months (Fig. 5b; Table 6b). These findings indicate that *T. retortaeformis* infective larvae recovered drove the pattern observed in the cumulative analysis.

Discussion

This study was motivated by two previous observations: first, average air temperature from our study site in Scotland has been on the rise over the last 30 years and second, during the same period and for a rabbit population from this field site, the intensity of infection of *G. strigosum* but not *T. retortaeformis* has been positively related to this climatic trend (Hudson *et al.*, 2006; Harvell *et al.*, 2009). To examine our prediction that climate warming equally affected the free-living stages of both parasite species, and thus that the contrasting long-term patterns observed were mainly driven by differences in host-parasite interactions, the effect of climate changes on the dynamics of free-living stages was examined in the laboratory and the field. Findings showed that the higher the amount of accumulated thermal energy the more eggs hatched, they hatched earlier and, for the field manipulations, more infective larvae were available on the pasture. The two parasite species exhibited analogous behaviors when perturbed by diverse temperature regimes, decadal trends or monthly temperatures although *T. retortaeformis* was more successful than *G. strigosum*, and this pattern was consistent in the laboratory and in the field. Importantly, we showed that seasonal changes in the intensity, frequency, and duration of daily temperature are a critical source of variability in egg hatching and larval survival and a significant contributor to the nonlinear relationship between climate and parasite dynamics. These patterns supported the general hypothesis that climate warming can enhance development and abundance of free-living stages of soil-transmitted helminths and potentially increase the force of infection and the risk of seasonal transmission.

The higher amount of thermal energy accumulated in the Constant regimes explained the relatively higher rate of hatching observed in this climatic setting compared to the cyclic simulations. The general positive trend

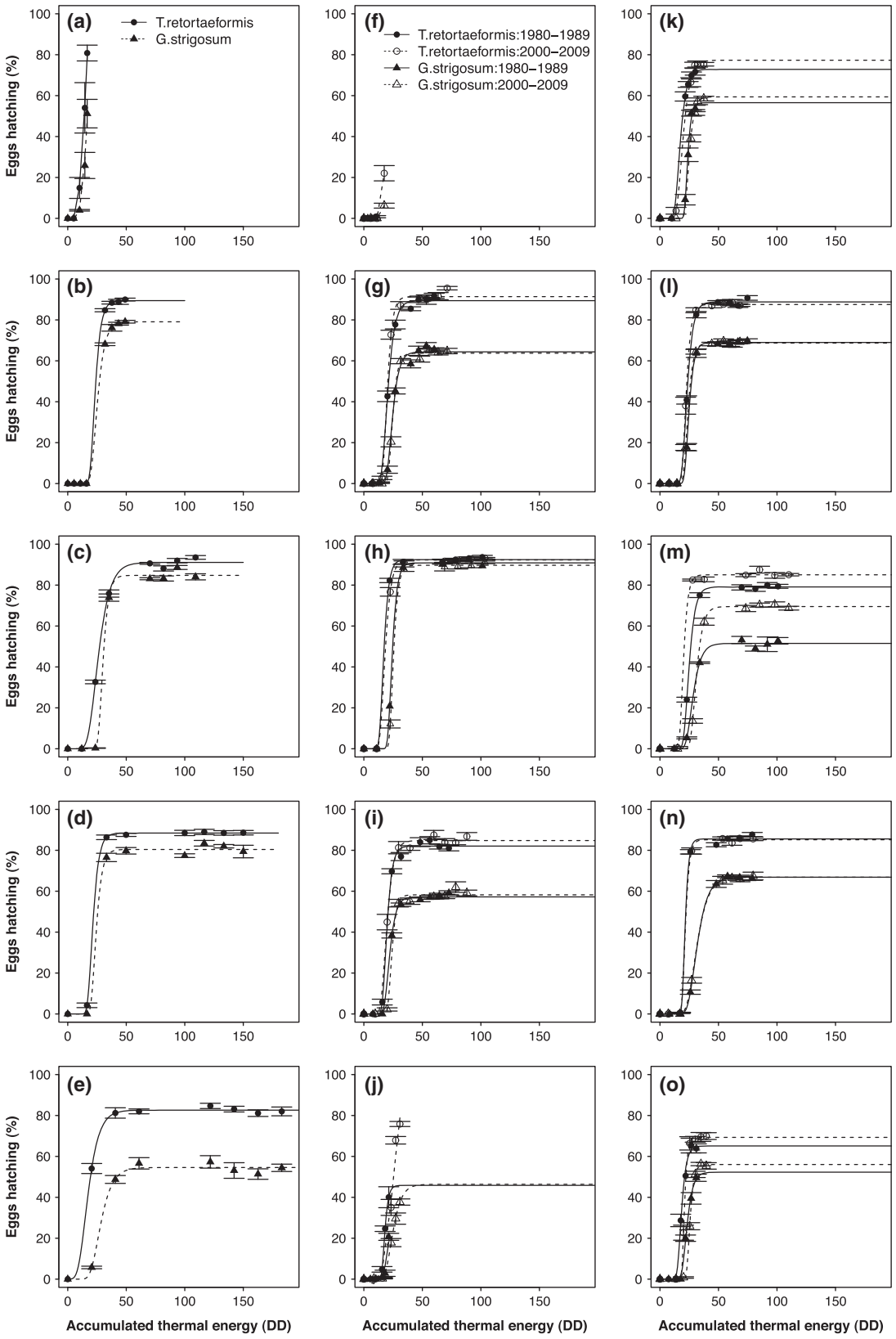

Fig. 3 Percentage of eggs that hatched by accumulated thermal energy in Constant regimes at 5°, 10°, 15°, 20°, and 25 °C, respectively (a–e), Cycle regimes in the months of March, May, July, September, and November, respectively (f–j), and Stochastic regimes in the months of March, May, July, September, and November, respectively (k–o). SE bars are reported.

Table 4 Linear mixed effects models between the proportion of eggs that hatched as a response variable, and month, decade and accumulated thermal energy (DD) as explanatory terms for: *Trichostrongylus retortaeformis* in Cycle (a) and Stochastic (c) regimes, and *Graphidium strigosum* in Cycle (b), and Stochastic (d) regimes

	(a)			(b)			(c)			(d)		
	Coeff. ± SE	df	*P*	Coeff. ± SE	df	*P*	Coeff. ± SE	df	*P*	Coeff. ± SE	df	*P*
Intercept	−0.07 ± 0.02	485	0.005	−0.01 ± 0.02	479	0.7	−0.15 ± 0.04	479	0.0003	−0.14 ± 0.03	484	<0.0001
May	0.3 ± 0.03	485	<0.0001	−0.03 ± 0.03	479	0.3	0.13 ± 0.06	479	0.02	0.12 ± 0.03	484	0.0004
July	0.2 ± 0.04	485	<0.0001	0.1 ± 0.03	479	0.004	0.26 ± 0.06	479	<0.0001	0.17 ± 0.03	484	<0.0001
September	0.14 ± 0.04	485	0.0001	0.02 ± 0.03	479	0.6	0.2 ± 0.06	479	0.0008	0.07 ± 0.03	484	0.05
November	0.06 ± 0.03	485	0.05	−0.05 ± 0.03	479	0.1	0.04 ± 0.06	479	0.5	0.03 ± 0.03	484	0.38
DD	0.01 ± 0.0004	485	<0.0001	0.0003 ± 0.0003	479	0.4	0.03 ± 0.002	479	<0.0001	0.02 ± 0.001	484	<0.0001
2000–2009							−0.08 ± 0.04	479	0.07	−0.02 ± 0.03	484	0.27
May*DD				0.01 ± 0.003	479	0.002	−0.01 ± 0.002	479	<0.0001	−0.01 ± 0.001	484	<0.0001
July*DD				0.01 ± 0.003	479	0.004	−0.02 ± 0.002	479	<0.0001	−0.01 ± 0.001	484	<0.0001
September*DD				0.01 ± 0.003	479	0.06	−0.02 ± 0.002	479	<0.0001	−0.01 ± 0.001	484	<0.0001
November*DD				0.01 ± 0.003	479	0.02	−0.01 ± 0.002	479	0.004	−0.004 ± 0.002	484	0.01
May*2000–2009							0.11 ± 0.06	479	0.054			
July*2000–2009							0.2 ± 0.06	479	0.0007			
September*2000–2009							0.05 ± 0.06	479	0.3			
November*2000–2009							0.05 ± 0.06	479	0.3			
DD*2000–2009										0.001 ± 0.0004	484	0.0002
Random factor: chamber	0.000006			0.01			0.000006			0.000008		
AIC	−0.13			−381.7			−59.8			−481.9		

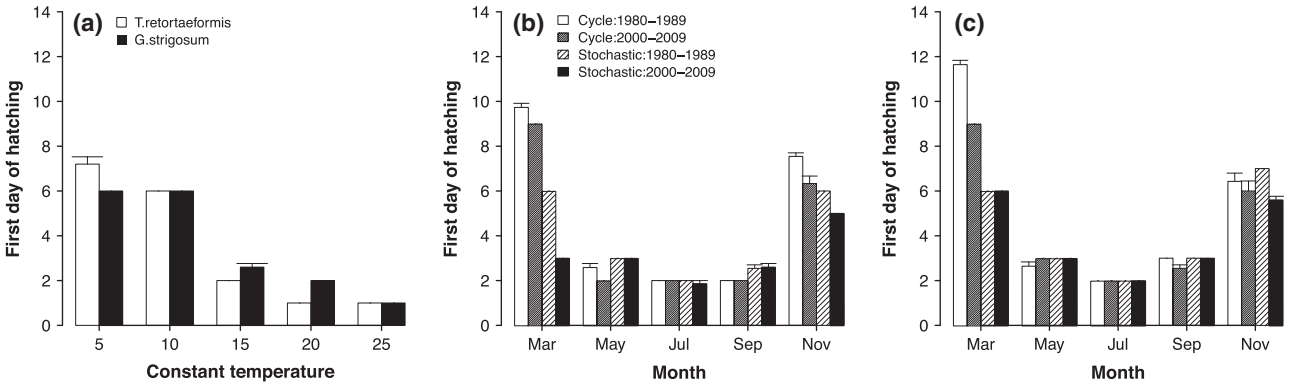

Fig. 4 First day of egg hatching in Constant regimes for both parasite species (a) and for *Trichostrongylus retortaeformis* (b) and *Graphidium strigosum* (c) by decades, months, and cyclic regimes. SE bars are reported.

Table 5 Linear mixed effects models between the average first day of egg hatching, as a response variable, and temperature regimes (Cycle and Stochastic), decade, and month for *Trichostrongylus retortaeformis* (a) and *Graphidium strigosum* (b)

	(a)			(b)		
	Coeff. ± SE	DF	*P*	Coeff. ± SE	DF	*P*
Intercept	2.16 ± 0.06	135	<0.0001	2.28 ± 0.03	132	<0.0001
May	−0.78 ± 0.05	135	<0.0001	−0.87 ± 0.04	132	<0.0001
July	−0.97 ± 0.05	135	<0.0001	−1.12 ± 0.04	132	<0.0001
September	−0.83 ± 0.05	135	<0.0001	−0.88 ± 0.04	132	<0.0001
November	−0.06 ± 0.05	135	0.2	−0.25 ± 0.04	132	<0.0001
Decade	−0.14 ± 0.05	135	0.006	−0.07 ± 0.02	132	0.007
Stochastic	−0.1 ± 0.03	135	0.004	−0.06 ± 0.02	132	0.01
Random factor: chamber	0.046			0.000006		
AIC	−26.1			−88.02		

between hatching and constant increase in temperature was comparable to previous studies on other Trichostrongylidae species (Hsu & Levine, 1977; Gibson, 1981; Beveridge *et al*., 1989; Pandey *et al*., 1989). Between the more realistic cyclic scenarios, the stochastic regimes accumulated greater thermal energy and caused higher rate of hatching than the day-and-night Cycles with fixed maxima and minima. Previous studies have demonstrated that fluctuations around a constant temperature resulted in higher hatching than fixed constant settings, indicating that variability in climatic conditions better stimulate the developmental and physiological properties of an individual (Hsu & Levine, 1977; Gibson, 1981; Saunders *et al*., 2002). Therefore, while the accumulation of thermal energy above some threshold is important for developmental time, equally important is the intensity and frequency of extreme thermal conditions that free-living parasite stages experience while on the herbage. One of the predictions in climate change studies is that the frequency of unpredictable extreme events will increase (Easterling *et al*., 2000). Our and work by others (Saunders *et al*., 2002) indicate that stochastic thermal events accelerate helminth development and the potential for higher transmission, assuming parasite survival remains the same (Dobson & Carper, 1992; Saunders *et al*., 2002; Hudson *et al*., 2006).

Across the 30 years of rising temperature recorded in our study site, cyclic simulations based on the two climatic decades showed that more eggs hatched in the recent warmer decade than the past colder period. This suggests that the long-term climate warming observed in the field may have had a general positive effect on the hatching of *G. strigosum* as well as *T. retortaeformis* eggs. This finding, and the previous observation that these two helminths exhibit contrasting dynamics of infection (Cattadori *et al*., 2005, 2008) and different relationships between infection intensity and temperature (Hudson *et al*., 2006), supports the initial hypothesis that the net effect of climate warming can be weakened in helminths that are mainly regulated by the host, for instance, when controlled by the immune system. Indeed, while rabbits develop a robust immune response to both parasites,

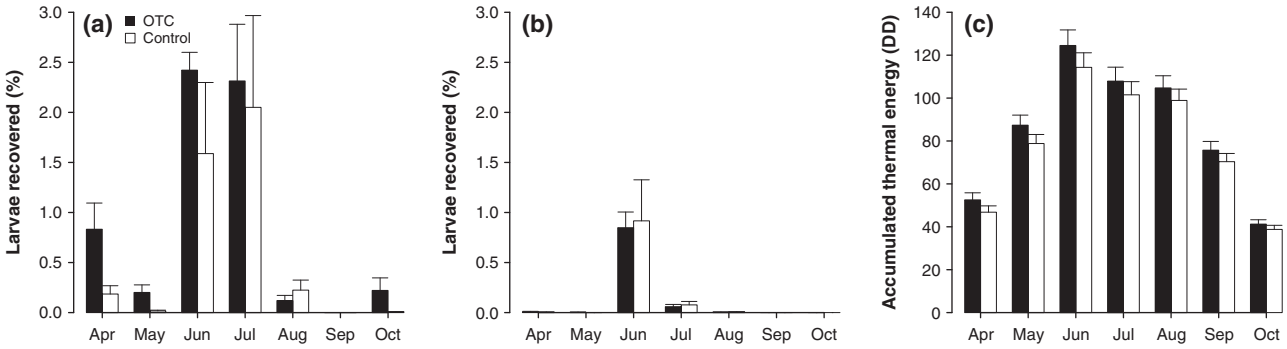

Fig. 5 Free-living infective larvae recovered in the experimental field manipulation for *Trichostrongylus retortaeformis* (a) and *Graphidium strigosum* (b) by sampling month and treatment, as well as the accumulated thermal energy (DD) by sampling month and treatment (c). SE bars are reported.

Table 6 General linear models (negative binomial error) between free-living infective larvae recovered in the field, as response variable, and treatment (control or OTC), and month of sampling for *Trichostrongylus retortaeformis* (a) and *Graphidium strigosum* (b)

	(a)		(b)	
	Coeff. ± SE	*P*	Coeff. ± SE	*P*
Intercept	−4.79 ± 0.33	<0.0001	−9.65 ± 0.59	<0.0001
April	−1.32 ± 0.46	0.004	−19.13 ± 4914.8	0.9
June	−1.42 ± 0.51	0.005	−1.37 ± 1.01	0.2
July	1.07 ± 0.46	0.022	4.92 ± 0.61	0.0001
August	1.02 ± 0.46	0.025	2.36 ± 0.62	<0.0001
September	−1.94 ± 0.48	<0.0001	−0.13 ± 0.93	0.9
October	−8.45 ± 3.03	0.005	−19.13 ± 4914.8	0.9
Treatment (Treat)	−1.5 ± 0.49	0.002		
Treat*April	−1.942 ± 0.85	0.02		
Treat*June	−0.989 ± 1.05	0.3		
Treat*July	1.079 ± 0.68	0.111		
Treat*August	1.381 ± 0.66	0.04		
Treat*September	2.126 ± 0.68	0.002		
Treat*October	2.195 ± 3.73	0.6		
AIC	460.43		151.62	

they can control *T. retortaeformis*, but not *G. strigosum*, with the latter showing a significant positive relationship with temperature (Cattadori *et al*., 2005, 2008; Murphy *et al*., 2011). An alternative explanation is that larval mortality increases proportionally with temperature, but occurs at much faster and higher rate for *T. retortaeformis* than *G. strigosum*. However, this is not supported by the field manipulations where we found that larval survival augmented in the summer months in the warmer OTCs for both parasites.

The two helminth species have identical free-living stages and transmission strategies, they are exposed to similar environmental elements in the field and are common infections of the same host. Yet, *T. retortaeformis* did much better than *G. strigosum* under similar climatic scenarios. Although this could be partly explained by phylogenetic differences between the two parasite species (Audebert & Durette-Desset, 2007), we do not exclude that the contrasting interactions with the host influenced hatching success directly, for instance, through the host immune response altering the quality of eggs shed in rabbit feces, or indirectly, by parasite demographic or immune processes affecting female worm conditions.

Both laboratory and field manipulations showed that monthly changes in thermal energy accumulation strongly influenced the seasonal dynamics of free-living stages. As expected, egg hatching and larval survival were at the highest in summer compared to spring and autumn months, supporting the high risk of infection frequently recorded during the spring-summer months

in animal populations exposed to soil-transmitted helminths from temperate areas (Pandey, 1974; May & Anderson, 1979; Smith *et al*., 1987; Anderson, 2000; Cattadori *et al*., 2005; Cornell *et al*., 2008). We also confirmed that parasite hatching is low to negligible during the cold months in this temperate area, in accordance with previous studies on other helminths species (Crofton, 1948b; Prasad, 1959; Gupta, 1961; Rogers & Sommerville, 1963; Stromberg, 1997). Data from simulations highlighted significant differences in the monthly temperatures recorded between the warm and the cold decade, however, no consistent differences were observed in the monthly rate of hatching between these two periods, with the exception of *T. retortaeformis* in July and partially May during the Stochastic regime. The difficulty to detect a clear long-term 'warming-effect' on parasite hatching at the monthly level was probably caused by using close, although statistically different, average monthly temperatures that lead to relatively similar hatching rates between months from different decades. It is also important to note that monthly decadal data were averaged over 10 years of field records and no negative or close to zero minima were simulated in the climatic chambers, and thus the contribution of extreme months was probably smoothed across the decade. However, we did find a strong positive effect of long-term warming on the monthly onset of first hatching. In other words, eggs of both helminths hatched faster in the recent warmer decade and this pattern was more apparent in March and November. This laboratory observation was supported by field manipulations that showed enhanced larval survival in the OTCs than the controls in the spring months suggesting that favorable climatic condition at the onset of the parasite growing season can extend the period of parasite activity and host exposure to infection.

The reduced hatching rate of *T. retortaeformis* and *G. strigosum* in the Constant 25 °C simulation raises the question of whether this temperature approaches the maximum thermal tolerance for egg development and hatching of these two parasites. Previous laboratory studies showed that *T. retortaeformis* egg development and hatching increased linearly up to 30 °C at 100% relative humidity, but that survival of infective larvae began to decrease above 25 °C (Prasad, 1959; Gupta, 1961). Similarly, relatively high temperatures, but low relative humidity in field experiments reduced larval survival for this species (Crofton, 1948b). Although comparable studies for *G. strigosum* are not available, infections in populations of rabbits from relatively warmer and drier regions are conspicuously lower when compared to more humid regions of Australia and New Zealand (Bull, 1964; Dunsmore, 1966). This suggests that too much thermal accumulation and extended dry seasons can increase larval mortality or delay egg hatching and thus reduce the risk of infection. Thus, warmer temperatures can lead to a net increase in transmission only if increases in development rates and productivity of parasites can outpace increases in mortality rates (Lafferty, 2009).

Despite the large volume of laboratory experiments and complementary field manipulations, a few limitations to our approach can be identified. First, the design of the laboratory trials prevented us measuring the survival of infective larvae as the medium precluded the development of first stage larvae into infective larvae and the experimental design was too short to measure long-term larval survival. Second, we did not examine the quality of the eggs used in our experiments, including controlling for the possible effects of the host immune response or age of infection on egg viability. However, we regularly mixed rabbit feces with different levels and duration of infection to reduce possible confounding effects associated with the host immune response and the parasite age. Third, experiments were run in three climatic chambers and we are aware that this may have increased between-experiment variability, yet, we corrected for this by including the chamber as a random error term in our models. Fourth, the majority of the laboratory experiments were executed only one time, however, a few trials repeated multiple times showed similar results suggesting that our findings are reliable and robust. Fifth, we only focused on temperature and more work needs to be done on the effect of relative humidity or photoperiod as they are known to be important for hatching of helminth eggs and survival of their larvae (Rogers & Sommerville, 1963; Croll, 1966; Langrova & Jankovska, 2004; Lutzelschwab *et al*., 2005; Micieli *et al*., 2012). Sixth, the limited availability of fresh feces/eggs affected the field manipulations and the likelihood of recovering a reliable number of eggs and larvae from the turf-grass squares. Although this experiment was replicated in two successive years, only the second time produced enough data that could be statistically analyzed.

In summary, parasites like other poikilothermic animals adapt life history strategies with responses optimized around temperature ranges that maximize survival and facilitate a higher reproductive potential. The response to a rise in temperature by free-living stages of related parasite species may be similar in direction, but with some variability depending on the physiological thresholds of each species. This study supports the prediction that by accelerating parasite development, climate warming can augment the availability of infective larvae on the pasture and the risk of seasonal transmission. In temperate regions, this

may represent a serious threat for livestock and wildlife seasonally exposed to parasites with similar life history traits. Yet, the parasite's success of infection will ultimately depend on the interaction between the host and the parasite. This is important for our understanding of relationships between climate changes and individual life history strategies and ultimately, population dynamics and long-term persistence. Future research is needed to examine how climate changes influence processes at the host-parasite interface and how multiple weather variables affect such interactions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Long-term climatic pattern from the field site.
Data S2. Average daily temperatures in Cycle and Stochastic regimes simulated in laboratory chambers.
Data S3. Comparison of climatic data between sampling sites.
Data S4.Estimation of the minimum biological threshold temperature (T_t) necessary for helminth egg development.
Data S5. Laboratory experiment analyses.
Data S6. Field experiment analysis.