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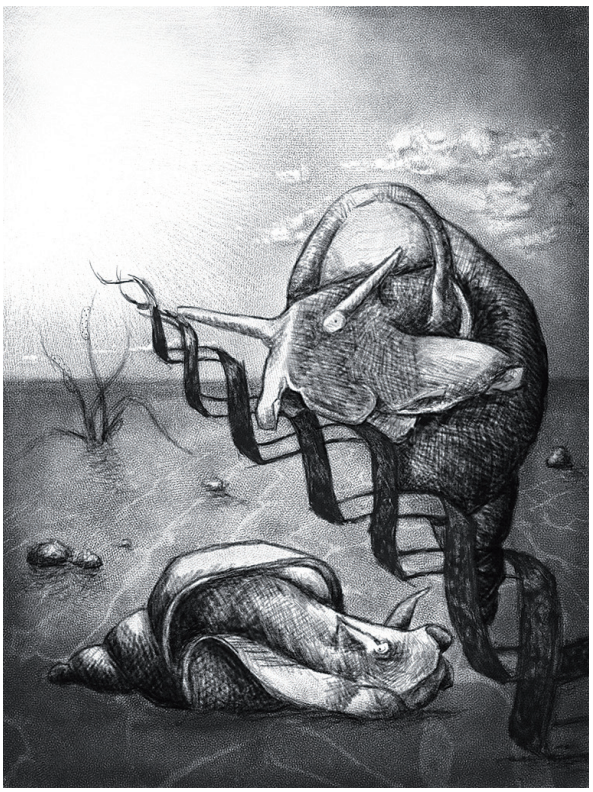
281

Katja Leicht

Implications of Heat Waves on Immune Defence, Life History Traits, and Adaptive Potential

A Snail's Perspective



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ABSTRACT

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Heat waves will increase in frequency and severity due to global climate change and can significantly alter organisms' ecology and evolution. In this thesis, I investigated the effects of experimental heat waves (25°C vs. 15°C) on immune defence and life history traits of the freshwater snail *Lymnaea stagnalis,* their cross-generational effects, and consequences for the interaction with a trematode parasite. I also examined the role of genetic factors (i.e. inbreeding, genetic variation, genetic covariation) in determining the effects of high temperature to assess potential limitations they cause for natural populations and their adaptive potential to climate change. I found that short-term heat waves altered investment in fitness-related traits, increasing overall performance of snails while long-term heat waves reduced it. The effects of heat waves on snails were transmitted across generations, benefitting early offspring traits but reducing later ones. Furthermore, high temperature increased overall infection success of a trematode parasite *Echinoparyphium aconiatum* in snails. This was determined by temperature effects on host traits other than immune function and/or on the parasite that overrode the effects of reduced immune defence. Regarding potentially limiting genetic factors, I found inbreeding depression in life history traits of snails, but this was not environment-dependent. I detected abundant genetic variation in immune defence and life history traits both at benign and high temperature, as well as in the response of one trait to high temperature (a genotype by environment interaction). Negative genetic covariation was scarce among traits and should not strongly limit their evolution. My results indicate that heat waves have strong direct and cross-generational effects on snails, and are likely to increase disease outbreaks in snail populations. However, snails show adaptive potential to climate change.

Keywords: Climate change, cross-generational effects, genetic covariation, genetic variation, host-parasite interaction, inbreeding, phenotypic responses.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-V.

I was responsible for the planning and execution of all studies, in cooperation with the coauthors. I collected the data together with Otto Seppälä for studies I, II, III, and IV, and with Otto Seppälä and Katri Liljeroos for study V. I conducted the data analyses together with Otto Seppälä for studies II, III, and V, and with Otto Seppälä and Jukka Jokela for studies I and IV. I had the primary responsibility of preparing all manuscripts.

I Leicht, K., Jokela, J. & Seppälä, O. 2013. An experimental heat wave changes immune defense and life history traits in a freshwater snail. *Ecology and Evolution* 3: 4861-4871.

II Leicht, K. & Seppälä, O. Direct and cross-generational effects of an experimental heat wave on early life stages in a freshwater snail. Manuscript.

III Leicht, K. & Seppälä, O. 2014. Infection success of *Echinoparyphium aconiatum* (Trematoda) in its snail host under high temperature: Role of host resistance. *Parasites & Vectors* 7: 192.

IV Leicht, K., Jokela, J. & Seppälä, O. Does inbreeding alter the effects of heat waves in a freshwater snail? Manuscript.

V Leicht, K., Liljeroos, K. & Seppälä, O. Adaptive potential under climate change: Family-level variation in fitness-related traits and their responses to heat waves in a snail. Submitted manuscript.

1 INTRODUCTION

1.1 Heat waves can alter animal lives

During the last hundred years, the intensity of global warming has become increasingly evident and it has been repeatedly shown to pose an extraordinary challenge for natural populations (Walther *et al.* 2002, Parmesan 2006, Walther 2010). Nowadays, research on the impact of climate change on natural systems is not only centered on the gradual increase in temperature, but the attention directs also to extreme weather conditions such as summer heat waves. Models widely agree that the frequency and severity of heat waves will increase in the future (Easterling *et al.* 2000, Meehl and Tebaldi 2004, Schär *et al.* 2004, IPCC 2013). Heat waves can have major consequences for individuals, populations, biodiversity, and species interactions, and they have been suggested to have an even greater impact in nature than the increase in average temperature (Hance *et al.* 2007, Walther 2010). As most abiotic disturbances, extreme temperature poses a severe challenge that leads to changes in optimal life history tactics (Baldwin and Dingle 1986, Partridge *et al.* 1995, Adamo and Lovett 2011). To optimize lifetime reproductive success it might become necessary to reallocate available resources between maintenance (e.g. immunocompetence) and reproduction (Hoffmann and Hercus 2000, Stearns 2000, Stefano *et al.* 2002). This, however, is only possible within physiological and biochemical boundaries set by extreme temperature (e.g. protein functioning, membrane fluidity) that can strongly reduce performance of an organism under such conditions (Angilletta 2009).

Individuals of ectothermic species may be especially sensitive to high temperature (Hance *et al.* 2007, Angilletta 2009). As an immediate effect, phenotypic changes in fitness-related traits may occur (de Jong 1995, Chevin *et al.* 2010). Plastic responses involve rapid mechanisms that can reversibly alter resource allocation among traits according to their relative importance under challenging conditions (Fisher 1930, DeWitt *et al.* 1998). Expression of fitness-related traits is subject to energetic trade-offs and the current investment in each

trait depends on intrinsic (e.g. condition, age) and extrinsic (e.g. environmental conditions, predator and parasite abundance) parameters (Fellowes *et al.* 1998, Marshall and Sinclair 2010, Cotter *et al.* 2011). Furthermore, observed changes in traits may result from an overall lack of available resources as extreme conditions can lead to an increased requirement of (specific) nutrients for maintaining body functions (Hofmann and Somero 1995, Chen *et al.* 2003, McNamara and Buchanan 2005). Those mechanisms may not be exclusive and the second mechanism may replace the first when challenging conditions such as exposure to high temperature are prolonged and physiological damage increases. Hence, predicting the effect of heat waves on individual performance and population level effects requires detailed knowledge of phenotypic changes in fitness-related traits under such conditions.

Changes in the phenotype of an individual due to environmental conditions can also be transmitted to the next generation via cross-generational effects (Mousseau and Fox 1998b). Cross-generational effects are non-genetic effects of the parental, most often maternal, phenotype on offspring phenotype that alter offspring performance (Mitchell and Read 2005, Janhunen *et al.* 2010). Maternal effects can be side effects of the altered physiological condition of the mother when she experiences challenging conditions (e.g. reduced investment in reproduction due to a lack of resources, exposure to stress hormones) and hence, decrease offspring performance (Kofman 2002, Steer *et al.* 2004, McCormick 2006). On the other hand, investment in reproduction by the mother might be increased, improving offspring performance (e.g. higher energy investment, provision of proteins to protect the embryo) when her metabolic rate and resource level is increased or when her reproductive value is shifted to current versus future reproduction under such conditions (Williams 1966, Marshall and Uller 2007, Liefting *et al.* 2010). Moreover, when the mother can match the phenotype of the offspring to the environment they are about to experience, these effects can prepare the offspring for future conditions (Agrawal 2002, Marshall and Uller 2007). It is therefore important to consider the magnitude and direction of these cross-generational effects, versus the direct effects of environmental conditions, when estimating the impact of climate change in nature. Knowledge about the role of cross-generational effects and their relative contribution to responses of organisms to climate change and heat waves can help to understand how challenging environmental conditions will affect natural populations.

Responses in life history traits to challenging conditions that alter individual performance are likely to have consequences not only for population dynamics, but also for species interactions such as host-parasite interactions (Harvell *et al.* 2002, Altizer *et al.* 2006). Thermal extreme events are known to lead to disease outbreaks in natural populations and the ecological and economic consequences entailing such events can be severe (Thomas and Blanford 2003, Bruno *et al.* 2007). A potential reason for the disease outbreaks is alterations in immune defence of hosts due to high temperature, which can lead to increased parasite infection success (Roth *et al.* 2010, Murdock *et al.* 2012). However, high temperature may also affect other host traits than immune

function that are important in determining resistance. For instance, increased food processing at high temperature increases the release of metabolic products (Hofmann and Todgham 2010) that can be used as host finding cues by parasites (Haas *et al.* 1995a). This could lead to higher parasite infection success (Haas *et al.* 1995a, Seppälä *et al.* 2011). Additionally, survival and infectivity of parasites can be modified, increasing their infection success under high temperature conditions (Evans 1985, Meyrowitsch *et al.* 1991, Fried and Ponder 2003). Because of these temperature effects on host traits other than immune function and the simultaneous effect on the parasite, the role of immune function in determining the outcomes of host-parasite interactions under high temperature conditions is not well understood. More work is needed in order to predict possible consequences of climate change for host-parasite interactions.

1.2 Genetic properties may limit evolutionary responses to heat waves

The genetic properties of natural populations can determine the magnitude of the detrimental effects caused by heat waves. Inbreeding, the mating of closely related individuals or self-fertilization, can reduce the genetic diversity of populations and increase the level of genome-wide homozygosity (Charlesworth and Charlesworth 1987). The resulting accumulation of deleterious recessive alleles (i.e. genetic load) and their expression can lead to severe fitness losses in natural populations, for example, by altering key life history traits (e.g. growth, reproduction) (Keller and Waller 2002, Roff 2002b). This reduction in fitness is also known as inbreeding depression (Cheptou and Donohue 2011). Moreover, inbred individuals are expected to suffer more from exposure to challenging conditions because they can enhance the expression of the increased genetic load (i.e. increase in the overall number of recessive deleterious alleles expressed) (Bijlsma *et al.* 1999), and environmental tolerance may be reduced (i.e. reduced potential to respond to environmental variation) (Auld and Relyea 2010). Populations with a low effective population size (as it is the case for endangered species) are especially vulnerable to the environment-dependent expression of an increased genetic load as they typically show a high level of inbreeding (Hedrick and Kalinowski 2000, Willi *et al.* 2006). Populations and species, however, vary in the effect that inbreeding has on their fitness and on their responses to high temperature (Lande and Schemske 1985, Kärkkäinen *et al.* 1996, Carr and Eubanks 2002). Therefore, estimating the detrimental effects of inbreeding on the performance of individuals and on the responses of inbred individuals to temperature change is important when trying to understand population-level consequences of extreme weather events due to climate change.

On an evolutionary time-scale, population-level consequences of climate change depend strongly on the ability of organisms to genetically adapt to

changing conditions. The risk of severe fitness losses imposed by modification of selective pressure under high temperature (e.g. shift of seasonal timing, new parasite species, (Etterson 2004)) are attenuated as populations adapt to new environmental regimes. Such evolutionary response in natural populations requires heritable genetic variation in the traits under selection. In general, genetic variation in life history traits is considered to be abundant (Lynch and Walsh 1998). However, recent studies assessing adaptation in fitness-related traits under climate change conditions have not found any evolutionary responses (Gienapp *et al.* 2008, Hoffmann and Sgrò 2011). It is suggested by experimental and theoretical work that the amount of genetic variation may be environment-dependent (Hoffmann and Merilä 1999) and thus, understanding the genetic variation in one environment is not sufficient for drawing conclusions about the adaptive potential under natural (i.e. variable) conditions. If a challenging environment alters the expression of genetic variation, then natural populations may not be able to respond adequately to changes in the various selective pressures imposed by the climate change (Kellermann *et al.* 2009, Kelly *et al.* 2012). However, even if the traits under selection show sufficient genetic variation, a negative genetic covariation with other traits (i.e. the proportion of variance that a trait shares with another trait due to the influence from common genes) can constrain evolutionary response (Lynch and Walsh 1998, Walsh and Blows 2009). Similar to the expression of genetic variation, the strength and sign of genetic covariation are often context-dependent (Sgrò and Hoffmann 2004) which can also constrain evolutionary response under climate change (Etterson and Shaw 2001). Therefore, it is important to examine the adaptive potential in fitness-related traits under challenging conditions to assess the potential of natural populations to adapt.

In addition, organisms need to be able to respond to the selective pressure imposed by the variation in temperature itself. Extreme temperature can inflict performance losses by, for instance, denaturation of protein structure, changes in membrane fluidity, or alterations of optimal resource allocation (van Noordwijk and de Jong 1986). Hence, genetic variation in the response to the rapidly occurring temperature changes is required in natural populations to adapt and persist under climate change (Lande 2009). To estimate genetic variation in trait responses across different environments their reaction norms can be assessed. Reaction norms describe the trait values expressed by a genetic unit along an environmental gradient (Bowman 1972). Parallel reaction norms of genetic units in a population indicate no genetically based differences in responses to variation in environmental conditions (Bowman 1972). When reaction norms are not parallel, the genetic units differ in their response to environmental variation (genotype by environment, G × E, interaction) and therefore provide the potential to respond to changing environmental conditions by the means of natural selection (Bowman 1972, Lande 2009). Detailed analyses of reaction norms can provide a more complete understanding of evolutionary processes in natural populations.

1.3 Aims of the study

In this thesis, I aimed to increase the knowledge on effects of climate change on the performance of organisms and species interactions. I focused on extreme temperature events (i.e. heat waves) as they are considered to have a large impact on organisms and will become more frequent in future. I investigated the effects of experimental heat waves on fitness-related traits of adults, offspring performance, and parasite resistance in the freshwater snail *Lymnaea stagnalis* (Linnaeus 1758). Furthermore, I assessed the role of genetic factors (e.g. inbreeding, genetic variation) and their interaction with temperature in determining life history and immune defence traits of snails to estimate their importance for the effects of heat waves and adaptive potential under climate change. More specifically, I first examined the responses in immune traits (haemocyte concentration, phenoloxidase (PO)-like activity, and antibacterial activity of haemolymph) and life history traits (growth and reproduction) of snails when exposed to experimental heat waves of different durations. I asked whether these responses were due to altered resource allocation, or a general decline in performance (I). Second, I investigated whether the thermal environment of mothers altered offspring phenotype and how strong these effects were compared to the direct effects of high temperature on offspring performance (II). Knowing the consequences of experimental heat waves on immune defence of snails, I examined their effects on infection success of a trematode parasite *Echinoparyphium aconiatum* (Trematoda) (Dietz 1909) (III). Furthermore, I examined whether inbreeding predisposed snails to negative effects of high temperature (i.e. environment-dependent expression of the genetic load) (IV). Finally, I estimated the potential for evolutionary adaptations in snails under climate change by measuring genetic variation in life history and immune traits as well as genetic correlations among them in different thermal environments. To assess the genetic variation in response to temperature variation, I measured reaction norms of family-lines across these environments (V).

2 STUDY SYSTEM AND METHODS

2.1 The great pond snail, *Lymnaea stagnalis*, and its thermal environment

Lymnaea stagnalis is a freshwater snail that is widely found across the northern hemisphere. *Lymnaea stagnalis* is a hermaphroditic species that prefers to outcross when a mating partner is present (Puurtinen *et al.* 2007). Furthermore, *L. stagnalis* is capable of storing allosperm for several months (Boycott *et al.* 1930). Adult snails breed successfully when water temperature is above 10°C (Vaughn 1953). Snails oviposit clutches containing three to 200 eggs (own obs.) which they attach to stones or plant material below the water surface. Developmental time can lie between 11 and 90 days and strongly depends on the ambient temperature (Vaughn 1953). Snails are mature with a size of approximately 1.5 to 2 cm and can grow up to 4 cm shell length (Crabb 1929).

Snails inhabit ponds and lakes with stagnant or slowly flowing water. The habitats I collected snails from were exclusively small ponds with a water depth of maximum 2 – 3 m. Such ponds are characterized by the lack of a stabile thermal layering during the warm months of the year (Pichler 1939), thus experiencing homothermy, and organisms inhabiting them cannot avoid high temperatures by seeking cooler water layers (Pichler 1939).

Measurements of water temperatures in four different ponds in Zürich (47°23'N, 8°33'E; 47°37'N, 8°58'E; 47°40'N, 8°55'E; 47°49'N, 8°69'E) during the summer months (July and August) in 2010, 2011, and 2012 showed a temperature range from 11 – 23°C with a mean minimum temperature of 13°C and a mean maximum temperature of 21°C across all ponds and all years. Ponds did not differ more than 4°C in their monthly minimum and maximum temperatures within the same year. In all ponds, variations between day and night temperatures never exceeded 5°C. There was no heat wave event in Switzerland during the time when the measurements were taken. Data from U. Tobler (unpublished) show that during a warm period in 2009, temperatures rose above 25°C (maximum 36°C) in small ponds.

2.2 Experimental snails and temperature exposures

The snails used in the experiments came from stock populations that originated from ponds in Zürich, Switzerland (47°23'N, 8°33'E and 47°37'N, 8°58'E). Approximately 45 adult snails per population were collected to start the stock populations in 2010 and in 2011. This number can be assumed to be sufficient to represent the genetic variation present in the natural populations. To avoid the loss of the genetic variation in stock populations, they were maintained in a population size of approximately 400 individuals. Populations were kept in water tanks that were connected to a biological filter, and fed with fresh lettuce. Water temperature in the tanks varied from 12°C to 20°C, but was around 15°C for most of the year.

In all experiments, snails were exposed to experimental heat waves of 25°C. This temperature was chosen as it is above the optimum temperature for snails (Vaughn 1953), but can occur during hot weather periods in summer (see above). 15°C was used as a control temperature as this is a common temperature in natural habitats of snails (see above).

Exposures to experimental heat waves took place in climate rooms set to either 25 or 15°C (± 1°C). Prior to the experiments, snails were maintained in plastic cups filled with 200 ml of aged tap water at 15°C for three to five days and fed *ad libitum* with spinach or lettuce to acclimate them to the experimental conditions. During the exposures, snails were maintained in a similar way in the climate rooms. Water in the cups was exchanged regularly by hand (I, juveniles in II) or snails were maintained in a flow through system (adults in II, III, IV, V).

2.3 Reproductive output as a measure of fitness

The reproductive output of snails during the experiments (I, II, IV, V) was assessed as a measure of fitness. Egg clutches were removed from the cups and pictures were taken from each clutch using a digital camera (Fujifilm FinePix F30, scene mode: close up, focal length: 35 mm, aperture: F/2.8, shutter speed: 1/85, sensitivity: ISO-200, image size: 2848 × 2136 pixels, focus mode: auto focus). In studies I and II, the eggs in each clutch were counted from the images. In studies IV and V, the number of eggs was estimated by measuring the area containing ten eggs from the images using ImageJ software (ImageJ 1.42q, Wayne Rasband, National Institute of Health, USA). Then, the area containing all eggs was measured and the number of eggs in the clutch was calculated.

2.4 Invertebrate immune system and immunological measurements

The invertebrate innate immune system is built by constitutive immune parameters that are constantly present in the haemolymph. The constitutive parameters are non-specific and form the first physiological barrier against a variety of parasites (Janeway *et al.* 2005). Intruding organisms can then activate the inducible part of the defence system which forms a second, more specific line of defence (Söderhäll 2010). In this thesis, assays were used that evaluate performance of the constitutive immune system, haemocyte concentration, phenoloxidase (PO)-like activity, and antibacterial activity of snail haemolymph. Haemocytes are phagocytic active digesting non-self particles, and represent the major part of the cellular immune response in invertebrates (van der Knaap *et al.* 1993). Furthermore, haemocytes also synthesize pro-phenoloxidase (pro-PO). When activated, PO catalyzes oxidative defence against parasites (Cerenius and Söderhäll 2004). Antimicrobial peptides are a further component of the innate immune defence and are active against invading microorganisms (Imler and Bulet 2005). These parameter are important for immune defence of most invertebrates including molluscs (e.g. Mitta *et al.* 2000, Butt *et al.* 2006, Hellio *et al.* 2007, Seppälä and Leicht 2013). In *L. stagnalis*, these immune parameters respond to various immune elicitors and hence, are involved in defence against infections (Seppälä and Leicht 2013).

Haemolymph samples for immunological measurements (I, IV, V) were taken by gently tapping on the foot of the snail with a pipette tip until it released haemolymph (Sminia 1981). From the haemolymph samples, haemocyte concentration, PO-like activity, and antibacterial activity were measured. Haemocyte concentration was counted immediately using a Neubauer haemocytometer (Blau Brand, Wertheim, Germany). To prevent haemocytes from sticking together, haemolymph samples (7 µl) were mixed with EDTA (4 mg/ml in H_2O, 7 µl). For later measurements of PO-like activity (10 µl haemolymph mixed with 100 µl PBS buffer) and antibacterial activity (70 µl pure haemolymph), samples were frozen in liquid nitrogen and stored at -80°C.

PO-like activity was measured spectrophotometrically (plate readers: Infinite 200; Tecan, Salzburg, Austria (I, V) and SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA (IV)) as the increase in optical density due to the oxidation of L-Dopa to dopachrom which is catalysed by PO. Haemolymph samples were thawed and centrifuged with 4000 g for 15 min. Of each sample 20 µl of the supernatant were taken and mixed with 140 µl cold, distilled water and 40 µl cold PBS buffer in the wells of a microtiter plate. Then, 20 µl L-Dopa (4 mg/ml in H_2O) were added in each well. In the controls, the haemolymph sample was replaced by distilled water to assess non-enzymatic oxidation of L-Dopa. The optical density was measured at 480 nm immediately when L-Dopa was added and again after 6 h incubation period at 30°C. The change in the

optical density was calculated as the difference between the two measurements. The mean of the five controls per plate was subtracted from each value of the same plate.

Antibacterial activity was measured spectrophotometrically (plate readers: Infinite 200; Tecan, Salzburg, Austria (I, V) and SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA (IV)) as the decrease in optical density due to the destruction of bacterial cells. Samples were thawed and 50 µl haemolymph were mixed with 200 µl of a bacterial solution containing lyophilized *Escherichia coli* ((Migula 1895) Castellani and Chalmers 1919) cells (Sigma Aldrich, 0.35 mg/ml in sodium phosphate buffer, pH 6) in the wells of a microtiter plate. In the controls, the haemolymph sample was replaced by distilled water as controls. The change in optical density was measured at 450 nm immediately after adding the bacterial solution and again after 30 min. The decrease in optical density was calculated as the difference between the measurements. The mean of the controls was subtracted from each value.

2.5 The trematode parasite, *Echinoparyphium aconiatum*, and experimental animals

In its natural habitat, various parasite species, including several virulent trematodes, use *L. stagnalis* as an intermediate host (Väyrynen *et al.* 2000, Faltýnková *et al.* 2007). Trematodes castrate the snails and can increase their mortality (Karvonen *et al.* 2004). Hence, life history traits and population dynamics of snails can be strongly affected by trematode infections (Brown *et al.* 1988, Sørensen and Minchella 1998).

The trematode *Echinoparyphium aconiatum* is a common parasite of *L. stagnalis* that uses the snail as first and second intermediate host (Loy and Haas 2001, Yurlova *et al.* 2006, Faltýnková *et al.* 2007). The definitive hosts are snail-eating birds (Alishauskaite 1960). Eggs are excreted into the water from birds with faeces and hatch into free-swimming miracidiae larvae (Huffman and Fried 2012). Miracidiae infect snails (first intermediate host) and develop into sporocysts in their gonads. Sporocysts reproduce asexually and produce rediae (Riech 1927, Huffman and Fried 2012). Growth of parasites in the gonad tissue leads to castration of the snail (Zbikowska 2006). Rediae produce cercariae which swim freely in water and actively search for another snail individual (second intermediate host) using chemical host cues (Haas 1994, Haas *et al.* 1995a). After contact, cercariae enter the host through the urinary orifice and develop into encysted metacercariae mostly in the hepatopancreas. When an infected snail gets eaten by a bird, parasites mature and reproduce sexually (Riech 1927).

In the infection experiment (III), parasite cercariae released by snails collected from ponds in Biengarten, Germany (49°39'N, 10°49'E) were used. *Echinoparyphium aconiatum* infections were identified by the morphology of the

cercariae. They can be recognized by their strongly developed gut system and the detailed structure of their collar spines (for morphological details and key see Faltýnková *et al.* (2007)).

3 RESULTS AND DISCUSSION

3.1 Effects of heat waves on snails and their interaction with a trematode parasite

Environmental variation, and in particular the unpredictable occurrence of extreme weather events such as summer heat waves, can impose a major challenge for populations of many species (Easterling *et al.* 2000, Montoya and Raffaelli 2010). Exposure to high temperature can result in altered population dynamics and species interactions, and may affect evolutionary responses of organisms under climate change (Parmesan and Yohe 2003, Parmesan 2006, Walther 2010). I found that in *L. stagnalis*, exposure to experimental heat waves altered fitness-related traits within and across generations (I, II), and snails' interaction with the trematode parasite *E. aconiatum* (III).

Exposure to high temperature had direct implications for life history and immune traits of *L. stagnalis* snails (I). Snails showed phenotypic responses to experimental heat waves in reproduction, growth, and immune parameters (haemocyte concentration, PO-like activity, antibacterial activity of haemolymph) (I, IV, V). These responses depended on the duration of the exposure to high temperature (I). Short-term exposures to experimental heat waves increased reproductive output and growth of snails while immune defence was maintained at a constant level (I). This could be due to an increase in the amount of available resources, probably through a faster metabolism and higher food intake at high temperature (Iguchi and Ikeda 1995, Person-Le Ruyet *et al.* 2004). The different responses of the examined traits to short-term exposure to high temperature indicate that the relative investment of resources among them was altered favouring growth and reproduction over immune function (Fisher 1930, Williams 1966, McNamara and Houston 1996). Under challenging conditions the optimal life history strategy may change in a way that higher investment in current reproduction enhances overall fitness. This can be due to a shift of the relative value of current reproduction (i.e. investment in reproduction) versus future reproduction (i.e. investment in self-

maintenance) when the risk of mortality is increased under challenging conditions (Fisher 1930, Williams 1966). Nevertheless, high temperature promotes the growth of (pathogenic) microbes in the water (White *et al.* 1991) and therefore an increase in immune defence could be expected. However, higher activity of the immune system can increase the risk of autoreactive self-damage (Read and Allen 2000, Sadd and Siva-Jothy 2006). A maintenance rather than an increase in immune activity could minimize the risk of reducing fitness through self-damage. Hence, exposure to short-term heat waves may not reduce but rather enhance the overall performance of snails which could lead to, for instance, an increase in population size.

In contrast, a prolonged duration (one week or longer) of the experimental heat wave decreased overall performance of snails (I). In detail, high reproductive rate and growth ceased, and constitutive immune defence was impaired (I). The overall reduction in performance indicates delayed physiological costs of high reproduction and growth of snails that is induced by the short-term response to high temperature. These costs may lay in temperature-induced damage of, for instance, proteins and cell membranes, and may require energetically costly protection and repair mechanisms to act (McNamara and Buchanan 2005). This can lead to an increased need for resources or to an under-supply with, for instance, micronutrients that are needed for tolerance mechanisms (Askew 1995, Chen *et al.* 2003). Hence, fewer resources are available for other traits leading to an overall decrease in performance (Sibly and Calow 1989). The negative effects detected here may significantly affect snail populations in future as the duration of heat waves is predicted to increase due to global climate change (Meehl and Tebaldi 2004, IPCC 2013).

These consequences of heat waves on snail populations may further be intensified. This is because phenotypic changes due to exposure to high temperature can, through their effects on life history decisions and on the physiological condition of individuals, be transmitted across generations (maternal and paternal effects) (Mousseau and Fox 1998b). Here, exposure of maternal snails to high temperature positively affected offspring performance in very early life stages (increased hatching success, slightly earlier onset of hatching) while their later performance was impaired (reduced survival after hatching, reduced size at the age of five weeks) (II). Challenged mothers may invest more in reproduction (e.g. energy resources, heat shock proteins) (Heath *et al.* 1999, Jann and Ward 1999, Steer *et al.* 2004), which can benefit especially very early offspring performance but not later one. This is because this maternal investment may deplete with the developing embryo, and the negative physiological condition of the mother may become apparent only later in offspring life (e.g. via maternal stress hormones offspring was exposed to) (Heath *et al.* 1999, McCormick 1999).

Development of eggs and early performance of offspring were largely impaired by direct effects of exposure to high temperature (reduced hatching success, strongly shortened developmental time, and reduced offspring survival, but increased size at five weeks) (II). Direct exposure of developing

embryos to high temperature can lead to a very short developmental time due to fastened biochemical processes. This, however, may lead to less well developed offspring at hatching and can reduce their survival (Pittman *et al.* 1990). Additionally, exposure of the very young stages of snails to high temperature can lead to protein denaturation and changes in membrane fluidity (Thorson 1950, Podolsky and Hoffmann 1998, Pörtner *et al.* 2005). This can affect the biochemical functioning of proteins and membranes and reduce the performance of snails. Interestingly, the magnitude of maternal effects on hatching success and survival of offspring was similar to the direct effect (II), indicating the importance of maternal effects for offspring performance. Hence, heat waves can have a strong impact across generations and this may also depend on at which life-cycle stage they occur. However, there was no evidence that offspring performance in one environment depended on the environment of the mother (II) as found in several species in response to different challenging agents (Fox *et al.* 1997, Räsänen *et al.* 2003, Marshall 2008). The evolution of maternal effects that explicitly prepare offspring for the environment they are about to experience requires reliable cues that enable mothers to predict future environmental conditions (Mousseau and Fox 1998a). Thus, the unpredictability of heat waves may prevent the evolution of such maternal effects. Overall, maternal effects due to heat waves are important to consider when investigating the impact of climate change on natural populations.

The individual and population level effects of climate change are also likely to have extending effects across interacting species. In particular, as high temperature affected immune defence traits in snails (I, II) this could alter their interaction with parasites (Harvell *et al.* 2002, Parmesan 2006, Hance *et al.* 2007). Accordingly, exposure to high temperature altered the interaction of *L. stagnalis* with its trematode parasite *E. aconiatum* by increasing the infection success of the parasite (III). Parasite infection success at high temperature was increased independently of the duration snails were maintained at challenging conditions before the exposure to the parasite. When controlling partly for the temperature-dependent changes in host metabolic and physiologic rate, and for the direct temperature effects on the parasite, infection success was only increased when snails were maintained at high temperature for a longer period before the exposure to the parasite (III). These results indicate that high temperature can decrease resistance of snails to parasites also independently of temperature effects on immune defence. This can be due to at least two different mechanisms. First, the increase in the metabolic rate in snails at high temperature can lead to a higher amount of released metabolic products (Iguchi and Ikeda 1995, Person-Le Ruyet *et al.* 2004). As parasites are known to use them as chemical cues for host finding (Haas *et al.* 1995a, Haas *et al.* 1995b, Körner and Haas 1998), this can enhance the encounter probability of parasite and host. Second, high temperature can directly affect parasites by increasing swimming speed or infectivity of cercariae which is known from closely related trematode species (Evans 1985, McCarthy 1999b, Morley 2011). Hence, these results show that already short-term heat waves, which do not reduce immune

function (I), can alter host-parasite dynamics due to the overriding effect of temperature on other host traits than immune function and/or on the parasite. This can lead to higher parasite prevalence in the population and a higher risk of disease outbreaks caused by heat waves.

3.2 Genetic properties and adaptive potential under heat waves

Genetic properties of individuals and populations can interact with environmental conditions in determining performance of organisms. This can potentially increase fitness losses and limit evolution under climate change (Skelly and Freidenburg 2001, Willi *et al.* 2006). I detected a negative effect of inbreeding on life history traits, but this did not depend on the temperature (IV). Moreover, snails should be able to adapt to extreme temperature since I found abundant genetic variation in fitness-related traits, but no strong negative genetic correlation among them under high temperature (V). However, the amount of genetic variation in responses of snails to high temperature (i.e. reaction norms between temperatures) was limited (V).

In general, the genetic properties of individuals are important in determining their performance. Hence, performance may be reduced by an increase in the genetic load (i.e. accumulated recessive deleterious alleles) as it is found under inbreeding (Keller and Waller 2002, Roff 2002b). The magnitude of reduction in performance can further depend on the environmental conditions inbred individuals are exposed to (Bijlsma *et al.* 1999, Fox and Reed 2011, Reed *et al.* 2012). In snails, inbreeding depression was found as reduction in life history traits (reproduction and size) at both the benign and the heat wave temperature in one of the examined populations (IV). This indicates that the increased genetic load reduces performance of inbred snails (Charlesworth and Willis 2009). Immune parameters as well as the responses of all examined traits to high temperature were not affected by inbreeding (IV). The lack of an inbreeding depression in these traits suggests that the genetic load may have been efficiently purged in the past. (Charlesworth and Charlesworth 1987, Bijlsma *et al.* 1999). The rate of purging is especially high under strong selection and hence, high selective pressure in the past may have let to efficient elimination of recessive deleterious alleles (Bijlsma *et al.* 1999). These results show that impaired genetic properties (i.e. increased homozygosity through self-fertilization) may reduce performance in life history traits in *L. stagnalis*. However, the effects of heat waves were not exacerbated by inbreeding in this experiment.

Some snails in this study voluntarily self-fertilized even though a potential mating partner was present, and their offspring had a high antibacterial activity at benign conditions (IV). The reason for the voluntary self-fertilization of snails is unknown and there are different possible explanations. First, snails that voluntarily self-fertilized and their potential mates may have been incompatible, preventing them from mating (Tregenza and Wedell 2000). High

self-compatibility of the snails may have led to high performance of their offspring (Hughes *et al.* 2009) as it is found in antibacterial activity. Second, it is possible that the potential mates of snails which decided to self-fertilize were infected with, for instance, pathogenic bacteria. That could lead to refusal to mate as well as to cross-generational immune priming increasing the level of constitutive immune defence traits in the produced offspring (Moret 2006, Sadd and Schmid-Hempel 2007). Hence, in both cases, the high antibacterial activity in snails produced through voluntary self-fertilization would not be a result from inbreeding.

Over evolutionary time-scales, genetic adaptation to changing environmental conditions may be the most effective mechanism allowing natural populations to persist under climate change. However, evidence for evolutionary responses to climate change is scarce, and recent studies report limited adaptive potential to climate change mediated environmental changes in natural populations (Gienapp *et al.* 2008, Kelly *et al.* 2012, Merilä 2012). Exposing snail families to benign and high temperature conditions revealed abundant genetic variation (i.e. adaptive potential) among families in life history and immune defence traits in both environments (V). This indicates that challenging thermal conditions do not limit the adaptive potential for these traits and hence, evolutionary adaptation by the means of natural selection should be possible. It further indicates that genetic variation is efficiently maintained, for instance, through limited local adaptation and frequent gene flow between populations (e.g. via migrants carried by water birds) (Sexton *et al.* 2011).

Nevertheless, negative genetic covariation would be another factor possibly limiting adaptation (Houle 1989, Atkins and Travis 2010). Genetic correlations among traits in snails exposed to different temperature treatments changed in their magnitude across temperatures but were mainly positive (V). Only one negative genetic correlation (between reproduction and antibacterial activity) appeared and that was under challenging conditions (V). This negative correlation indicates that, for instance, maintenance traits such as immune defence may be traded off against reproduction in order to maximize lifetime fitness under challenging conditions (Zera and Harshman 2001, Monaghan *et al.* 2009). Moreover, resource limitation under such conditions (e.g. through protection and repair mechanisms of cells and membranes) may also lead to trade-offs in resource allocation among traits (Lynch and Walsh 1998, Walsh and Blows 2009). However, as negative genetic correlations under high temperature were scarce (V), they may not significantly limit adaptation processes of immune and life history traits under climate change.

As temperature changes itself can impose selective pressure on organisms, genetic variation in responses to temperature variation during heat waves is crucial for evolutionary adaptation. Snail families did not show significant G × E interactions across the two thermal environments in most examined traits (V). Only one immune trait, PO-like activity, showed genetic variation in response to temperature variation (V). Parallel reaction norms across temperatures are found for many immune and life history traits and may limit

adaptive potential for temperature variation (Stearns 1992). However, in some studies there is evidence for genetic variation in response to temperature variation in thermal reaction norms of some life history traits (e.g. timing of reproduction, growth rate), similar to the significant G × E interaction found here (V). This indicates genetic potential for adaptive responses to high temperature (Brommer *et al.* 2005, Husby *et al.* 2010). Hence, even though G × E interactions were rare in this study, the one observed G × E interaction points out that the price snails need to pay for increased growth and reproduction by compromising immune defence (i.e. PO-like activity) has adaptive potential in response to high temperature. Taken as a whole, snails should be able to evolutionary respond to the various selective pressures which are modified under climate change and heat waves, and hence, may persist without difficulty over longer time scales.

4 CONCLUSIONS

The main goal of this thesis was to increase our understanding on the responses of organisms to climate change with the specific emphasis on heat waves. Using a freshwater snail *L. stagnalis*, I could show that heat waves altered life history and immune defence traits, first increasing reproduction and growth and, when heat waves became longer, decreasing performance in both life history and immune traits (I). These effects were transmitted to the next generation, benefitting very early offspring traits but reducing later offspring performance (II). I could further show that maternal effects on very early offspring performance were equally strong to the direct effects of high temperature (II). High temperature also affected the interaction between snails and the trematode *E. aconiatum* by increasing infection success of the parasite (III). However, I found that the temperature effects on the immune defence of snails in determining parasite infection success were overridden by other effects of temperature on hosts (e.g. increased release of chemical cues) and/or by direct effects of temperature on the parasite (e.g. increased infectivity) in determining infection success (III). Furthermore, even though I found some evidence for inbreeding depression, the responses of life history and immune traits to high temperature were robust against inbreeding, not further reducing their performance (IV). I could also show abundant genetic variation in life history and immune traits under both benign and high temperature conditions, and in response to temperature variation in one immune trait (i.e. G × E interaction in PO-like activity) (V). This adaptive potential was not strongly limited by genetic correlation among traits (V).

The mechanisms behind the changes found in fitness-related traits are life history decisions that are made within the physiological and biochemical constraints set by extreme temperature. Life history theory predicts that investment in traits is optimal for lifetime reproductive success (i.e. fitness) (Williams 1966, Stearns 1992, Roff 2002a). This optimal investment of resources among traits will change under challenging conditions when the fitness landscape changes and the reproductive value of an individual is altered due to potentially reduced longevity under such conditions (Stearns 1992, Roff 2002a).

Hence, investment in current reproduction increases under challenging temperature conditions within the boundaries set by the physiology of an organism (I, II). Simultaneously, maintenance traits, such as immune function, are traded off (I, III), and the risk of fitness losses through, for instance, parasite infections increases (III). On the other hand, temperature itself is a strong driver of performance in ectothermic species (Angilletta 2009). When temperature increases, it increases the metabolic rate but can also lead to reduced body function through a decreased biochemical functioning (Iguchi and Ikeda 1995, Person-Le Ruyet *et al.* 2004). This can be assumed to be at least partly responsible for changes in life history traits such as reproduction, growth rate, and mortality (I, II), as well as for the possibly increased release of chemical compounds used for host finding by trematode cercariae (III).

Hence, the effects of temperature on snails are the result of complex interactions among intrinsic (e.g. life histories, genetic properties) and extrinsic (e.g. species interactions) components, which could alter population dynamics and may reduce population size (Pörtner *et al.* 2005, Angilletta 2009). This reduces the genetic diversity of populations and can increase the level of inbreeding (Keller and Waller 2002). Even though inbreeding often reduces performance in life history traits (Frankham 1996), and may alter the response of organisms to challenging conditions (Cheptou and Donohue 2011), the negative effects of inbreeding can be eliminated by selection against deleterious recessive alleles (i.e. purging) (Bijlsma *et al.* 1999, Fox *et al.* 2011). This may prevent further changes in the responses of inbred organisms to extreme temperature (IV). Also adaptive evolution can allow populations to optimize their fitness despite extreme conditions and hence may buffer population declines over evolutionary time-scales (IV, V). Given that adaptation processes are not limited by low genetic variation and genetic trade-offs between life history traits (as indicated in study V), they provide an efficient response mechanism for natural populations under global climate change.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Lämpöaaltojen merkitys immuunipuolustukselle, elinkiertopiirteille ja sopeumien mahdollisuudelle – kotilon näkökulma

Maailmanlaajuinen ilmaston lämpeneminen on ollut voimakasta viimeisen sadan vuoden aikana ja lämpenemisen ennustetaan edelleen jatkuvan. Lämpenemisellä on havaittu olevan laajoja vaikutuksia monien lajien populaatioihin, luonnon monimuotoisuuteen ja ekosysteemien toimintaan. Keskilämpötilojen nousun lisäksi myös lämpöaallot yleistyvät, voimistuvat ja pidentyvät ilmastonmuutoksen seurauksena. Tällä on esitetty olevan jopa suurempia vaikutuksia lajien ekologiaan ja evoluutioon kuin keskilämpötilojen tasaisella nousulla. Väitöskirjatyössäni tutkin kokeellisesti lämpöaaltojen (25 °C vastaan 15 °C) vaikutusta yksilöiden immuunipuolustukseen ja elinkiertopiirteisiin, niiden sukupolven yli ulottuvia vaikutuksia, sekä vaikutusta loisalttiuteen käyttäen *Lymnaea stagnalis* makeanveden kotiloita. Tutkin myös geneettisten tekijöiden (sisäsiittoisuus, piirteissä havaittava geneettinen vaihtelu) merkitystä lämpötilan vaikutukseen selvittääkseni niiden luonnonpopulaatioille aiheuttamia rajoitteita ja mahdollisuutta sopeutua ilmastonmuutokseen.

Tutkimuksessani havaitsin, että lyhytkestoinen (alle viikon) altistus korkealle lämpötilalle lisäsi kotiloiden kasvua ja lisääntymistehoa, mutta ei vaikuttanut immuunipuolustukseen. Tämä voi johtua kotiloiden lisääntyneestä aineenvaihduntanopeudesta ja muuttuneesta resurssien jakamisesta eri piirteiden välillä. Pitkäkestoinen (vähintään viikon) lämpöaalto johti kuitenkin heikentyneeseen lisääntymiseen ja immuunipuolustukseen. Tämä osoittaa, että kotilot pystyvät ylläpitämään korkeaa suorituskykyä lyhytaikaisesti ja korkean lämpötilan vaikutukset muuttuvat altistusajan pidetessä haitallisiksi. Altistus lämpöaallolle vaikutti myös kotiloiden tuottamiin jälkeläisiin. Emojen altistus korkealle lämpötilalle lisäsi tuotettujen munien kehitysnopeutta ja kuoriutumistodennäköisyyttä mutta haittasi kuoriutuneita poikasia (hidastunut kasvu, alentunut selviytyminen). Kotilot saattavat siten panostaa enemmän tuotettujen jälkeläisten laatuun korkeassa lämpötilassa, mutta tämä ei riitä edistämään poikasten suorituskykyä kuin aivan varhaisimmissa elämän vaiheissa. Havaitut äitivaikutukset munien kuoriutumistodennäköisyydessä ja poikasten selviytymisessä olivat yhtä suuria kuin lämpötilan suorat vaikutukset poikasiin. Tulos korostaa sukupolvien välisten vaikutusten tutkimisen tärkeyttä ilmastonmuutoksesta johtuvien ympäristömuutosten seurauksia arvioidessa. Korkea lämpötila muutti kotiloiden vuorovaikutusta *Echinoparyphium aconiatum* imumatoloisen kanssa lisäämällä loisen infektiomenestystä. Tämä vaikutus ei kuitenkaan johtunut muutoksista kotiloiden immuunipuolustuksessa vaan todennäköisesti lämpötilan muista fysiologisista vaikutuksista kotiloihin tai loisen toukkavaiheisiin.

Kotiloiden geneettiset ominaisuudet vaikuttivat osittain lämpöaaltojen aiheuttamiin muutoksiin elinkierto- ja immuunipuolustuspiirteissä. Sisäsiittoisuus vähensi kotiloiden kelpoisuutta heikentämällä kasvua ja lisääntymistä

mutta ei lisännyt lämpöaaltojen haitallisia vaikutuksia kotiloihin. Sisäsiittoisuus on usein ongelma erityisesti pienissä ja eristyneissä populaatioissa. Tulosteni mukaan sisäsiittoisten kotilopopulaatioiden ei kuitenkaan voida olettaa olevan muita populaatioita alttiimpia lämpöaaltojen vaikutuksille. Tutkimuksessa havaitsin populaation sisäistä geneettistä vaihtelua perhetasolla sekä elinkierto- että immuunipuolustuspiirteissä molemmissa testatuissa lämpötiloissa. Lisäksi korkean lämpötilan negatiivinen vaikutus yhteen immuunipuolustuspiirteeseen (fenoloksidaasi-aktiivisuus) vaihteli perheiden välillä osoittaen geneettistä vaihtelua kotiloiden vasteessa lämpötilamuutoksiin. Havaittu geneettinen vaihtelu mahdollistaa piirteiden evoluution tulevaisuudessa. Geneettiset allokaatiokustannukset tutkittujen piirteiden välillä olivat harvinaisia, joten niiden ei voida olettaa rajoittavan kotiloiden evoluutiota voimakkaasti. Tutkimukseni tulokset osoittavat, että lämpöaalloilla on voimakkaita suoria ja sukupolven yli ulottuvia vaikutuksia kotiloihin. Lisäksi lämpöaallot voivat lisätä tautien yleisyyttä luonnon populaatioissa. Lämpöaallot eivät kuitenkaan rajoita kotiloiden elinkierto- ja immuunipuolustuspiirteiden mahdollisuutta evoluutioon, joten luonnon populaatiot saattavat pystyä sopeutumaan ilmastonmuutoksen seurauksena muuttuviin ympäristöolosuhteisiin.

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ORIGINAL PAPERS

I

AN EXPERIMENTAL HEAT WAVE CHANGES IMMUNE DEFENCE AND LIFE HISTORY TRAITS IN A FRESHWATER SNAIL

by

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An experimental heat wave changes immune defense and life history traits in a freshwater snail

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Abstract

The predicted increase in frequency and severity of heat waves due to climate change is expected to alter disease dynamics by reducing hosts' ability to resist infections. This could take place via two different mechanisms: (1) through general reduction in hosts' performance under harsh environmental conditions and/or (2) through altered resource allocation that reduces expression of defense traits in order to maintain other traits. We tested these alternative hypotheses by measuring the effect of an experimental heat wave (25 vs. 15°C) on the constitutive level of immune defense (hemocyte concentration, phenoloxidase [PO]-like activity, antibacterial activity of hemolymph), and life history traits (growth and number of oviposited eggs) of the great pond snail *Lymnaea stagnalis*. We also manipulated the exposure time to high temperature (1, 3, 5, 7, 9, or 11 days). We found that if the exposure to high temperature lasted <1 week, immune function was not affected. However, when the exposure lasted longer than that, the level of snails' immune function (hemocyte concentration and PO-like activity) was reduced. Snails' growth and reproduction increased within the first week of exposure to high temperature. However, longer exposures did not lead to a further increase in cumulative reproductive output. Our results show that short experimental heat waves do not alter immune function but lead to plastic responses that increase snails' growth and reproduction. Thus, although the relative expression of traits changes, short experimental heat waves do not impair snails' defenses. Negative effects on performance get pronounced when the heat waves are prolonged suggesting that high performance cannot be maintained over long time periods. This ultimately reduces the levels of defense traits.

Introduction

Temperature is one of the most important environmental factors affecting performance and life histories of organisms (Angilletta 2009). In particular, poikilothermic organisms depend strongly on ambient temperature and respond physiologically to temperature variation. While daily and seasonal changes in temperature are largely predictable and can be counteracted with acclimation processes (Kingsolver and Huey 1998; Sørensen and Loeschcke 2002), the unpredictable occurrence of extreme temperatures can seriously impair organisms' overall performance (Wegner et al. 2008; Roth et al. 2010; Roux et al. 2010). This is because rapidly acting protection mechanisms that increase thermal tolerance are energetically costly (see Feder and Hofmann 1999; Somero 2002 for review), and when temperature becomes too extreme, enzymatic function, membrane structure, and oxygen supply can be impaired (Pörtner et al. 2005). Therefore, extreme weather events are expected to cause severe perturbations in organismal function.

Such unpredictable extreme weather conditions (e.g., heat waves) are expected to become more frequent, more intense, and last for longer time periods in the near future due to global climate change (Easterling et al. 2000; Meehl and Tebaldi 2004; Diffenbaugh et al. 2005). As such events are known to modify many life history traits including organisms' growth rate, longevity, and

reproduction (Baldwin and Dingle 1986; Partridge et al. 1995; Parmesan 2006; Adamo and Lovett 2011), climate change may have wide implications on natural populations and ecosystems (see Sarmento et al. 2010; Walther 2010 for review, Montoya and Raffaelli 2010). In addition, extreme weather events may alter ecological interactions including predator–prey (see Parmesan 2006; McCluney et al. 2012 for review) and host–parasite interactions (see Harvell et al. 2002; Hance et al. 2007 for review, Mangal et al. 2008). For example, extreme temperature can increase parasite virulence (Poulin 2006; Wegner et al. 2008) and reduce host immune defense (Roth et al. 2010; Karl et al. 2011; Seppälä and Jokela 2011; Murdock et al. 2012).

Reduced immune function has been demonstrated as a response to several harsh environmental factors and can be due to two different mechanisms. First, host performance could be reduced overall, which would be seen as a decrease across a broad range of host traits (McNamara and Buchanan 2005; Marshall and Sinclair 2010). We call this a general reduction hypothesis. Second, natural selection could favor plastic responses that optimize trait expression by compromising defense traits in order to maintain other traits to maximize fitness under suboptimal conditions (Hoffmann and Hercus 2000; Stearns 2000; Stefano et al. 2002; Adamo and Lovett 2011). We call this an altered resource allocation hypothesis. Additionally, the relative role of the above mechanisms in altering hosts' performance may depend on severity (e.g., duration) of extreme conditions. Thus, in order to understand the mechanisms underlying impaired resistance and their relative importance, it is necessary to couple the observed changes in hosts' defense mechanisms and/or susceptibility to infections over time with simultaneous responses in other life history traits.

In this study, we tested these alternative hypotheses (general reduction vs. altered resource allocation) by investigating the effects of an experimental heat wave on a freshwater snail, *Lymnaea stagnalis*. In its natural habitats, *L. stagnalis* is predicted to experience increasingly intense heat waves in future (Schär et al. 2004). Furthermore, in this system, exposure to high temperature is known to impair the constitutive level of snails' immune function (Seppälä and Jokela 2011). We measured three immune traits important for snails' innate immune system (hemocyte concentration, phenoloxidase [PO]-like activity, and antibacterial activity of hemolymph). From life history traits, we chose to measure growth and reproductive output as they are strongly connected to fitness and commonly traded off with immune defense in other systems (Nordling et al. 1998; French et al. 2007). We chose 25°C as a high (i.e., heat wave) temperature as it lies above snails' thermal optimum (Vaughn 1953), but occurs intermittently in snails' habitats during hot summers (A. Laurila, 2010, unpubl. data; U. Tobler, 2010, unpubl. data). We used 15°C as a control temperature as it is close to snails' thermal optimum (Vaughn 1953) and common in ponds (A. Laurila, 2010, unpubl. data; U. Tobler, 2010, unpubl. data). We exposed snails to these temperatures for up to 11 days as the present-day observations from Western Europe show that heat waves last on average 8.40 days (Meehl and Tebaldi 2004). We predict that if the first hypothesis (general reduction) applies, we would observe an immediate decrease in both immune and life history traits. Alternatively, if the second hypothesis (altered resource allocation) applies, we predict that snails reduce expression of immune traits in order to maintain reproductive life history traits. Furthermore, if the relative role of the above mechanisms depends on the length of exposure to high temperature, we expect general negative effects to become more pronounced over time.

Material and Methods

Experimental animals

Lymnaea stagnalis is a hermaphroditic snail that is common in ponds and lakes throughout the holarctic region. In its natural habitats, *L. stagnalis* is an important host for various parasites including several highly virulent trematode species (Väyrynen et al. 2000; Faltýnková et al. 2007) that castrate the snails and increase their mortality (Karvonen et al. 2004). Snails used in this study came from a laboratory stock population (F_2 generation) originating from a pond in Zürich, Switzerland (47°22′N, 8°34′E). The population was maintained in water tanks (temperature ranging from 12 to 20°C, but being close to 15°C for most of the year) for 1 year before the experiment. All snails used in the experiment were at reproductive age. One week prior to the experiment, experimental snails were randomly chosen from the population and placed individually in plastic cups filled with 2 dL of aged tap water. Snails were maintained at 15 ± 1°C and fed ad libitum with spinach to acclimate them to the experimental conditions. During that time, half of the water in the cups was changed daily.

Experimental design

Experimental snails were maintained as described earlier, and each of them was randomly assigned into one of the two temperature (15 ± 1 and 25 ± 1°C) and to one of the six exposure time treatments (1, 3, 5, 7, 9, and 11 days; 30 snails per treatment combination. Initial size (i.e., shell length) of the snails (range: 24–42 mm) did not differ between the temperature and exposure time

treatments (analysis of variance [ANOVA]: $P > 0.05$ for both). At the beginning of the experiment, snails exposed to high temperature were slowly warmed up to 25°C (over 10 h). At the end of the experiment, snails' shell length was measured to the nearest 0.1 mm, and immune traits and reproduction were measured as described below. Snails' energy reserves were measured and analyzed as described in the Appendix S1. A total of twelve snails died during the experiment, and not all traits could be measured from 36 individuals. As the mortality of snails during the experiment was generally low (3.8%), survival could not be used as a response variable to examine the effects of experimental treatments. Therefore, these snails were excluded from the data.

Effects of temperature and exposure time on immune traits

Hemocyte concentration, PO-like activity, and antibacterial activity of snail hemolymph were measured to determine the functional response of snails' immune system to different temperatures. Measuring snail immune function rather than the susceptibility to some specific parasite species was chosen because examining several immune parameters gives a broad estimate of host defenses, whereas studies focusing on a certain host–parasite interaction are necessarily specific to the particular parasite species being used. Such studies can also be confounded by direct effects of temperature on parasite transmission stages (Haas 1994; Pechenik and Fried 1995; McCarthy 1999). Hemocytes, through phagocytosis, constitute the main part of the cellular immune response (van der Knaap et al. 1993). Hemocytes also synthesize prophenoloxidase (pro-PO), which in the active form PO catalyzes oxidative defense against parasites (Cerenius and Söderhäll 2004). Note that the amount of active PO in snail hemolymph, not the amount of zymogenic pro-PO was measured in this study. As a further component of the innate immune defense, antibacterial peptides are used against microorganisms (Imler and Bulet 2005). The measured immune parameters are important components of immune defense in invertebrates including mollusks, although they have also other functions (Butt et al. 2006; Hellio et al. 2007; e.g., Mitta et al. 2000; Seppälä and Leicht 2013). Also in *L. stagnalis*, these immune parameters are known to be involved in defense against infections as they respond to immune elicitors (Seppälä and Leicht 2013).

To collect hemolymph samples for the immune measurements, each snail was removed from its cup at the end of its temperature treatment (i.e., after 1, 3, 5, 7, 9, or 11 days of exposure), blot dried, and its foot was gently tapped to induce the expulsion of blood (Sminia 1981). Hemolymph samples to measure PO-like activity (10 μL of hemolymph mixed with 100 μL of phosphate-buffered saline [PBS, pH 7.4]) and level of antibacterial activity (100 μL of pure hemolymph) were snap frozen in liquid nitrogen and stored at −80°C for later analysis (see below). Fresh hemolymph was used to measure its hemocyte concentration (cells per μL). To prevent hemocytes from sticking together, 7 μL of hemolymph was mixed with 7 μL of EDTA solution (Sigma-Aldrich, Steinheim, Germany, 4 mg per ml in H_2O) before counting the cells using a Neubauer hemocytometer (Blau Brand, Wertheim, Germany).

At a later date, PO-like activity was measured with a spectrophotometer using a microtiter plate reader (Infinite 200; Tecan, Salzburg, Austria). Samples were thawed on ice and centrifuged at 4000 *g* for 15 min, after which the supernatant was collected. Each well of a microtiter plate was filled with 140 μL of cold distilled water, 20 μL of cold PBS, and 40 μL of a sample. After this, 20 μL of L-Dopa (4 mg/mL in H_2O) was added into each well. In the following reaction, the enzyme PO oxidizes L-Dopa to dopachrom, which leads to an increase in the optical density (OD) of the solution. In five wells per plate, the hemolymph sample was replaced with distilled water to control for nonenzymatic oxidation of L-Dopa. The OD was measured at 480 nm immediately after L-Dopa was added and again after a 6-h incubation period at 30°C (instrument measurement range 0–4 OD with 0 being completely transparent and 4 being nontransparent). During that period, the increase in OD over time is linear (O. Seppälä, 2007, unpubl. data). PO-like activity was calculated by subtracting the OD directly after adding L-Dopa from the OD after 6 h. Mean change in the OD of the controls was subtracted from all measurements, and change in OD was calculated in milliunits.

To measure the antibacterial activity of snail hemolymph, 50 μL of hemolymph was mixed with 200 μL of a solution containing lyophilized *Escherichia coli* cells (Sigma-Aldrich; 0.35 mg bacteria cells per ml sodium phosphate buffer, pH 6) in wells of microtiter plates at 20°C. In the reaction, antibacterial enzymes destroy *E. coli* cells. This leads to a decrease in OD of the solution over time. In five wells per plate, the hemolymph sample was replaced with distilled water to control for changes in OD not caused by antibacterial activity. OD was measured at 450 nm immediately after mixing the hemolymph and bacteria and again after 30 min using a microtiter plate reader (Infinite 200; Tecan). During that period, the decrease in OD over time is linear (O. Seppälä, 2007, unpubl. data). The decrease in OD was calculated by subtracting OD after 30 min from OD at the first measurement. Mean change in controls was subtracted from all measurements, and change in OD was calculated in milliunits.

All immune parameters were measured twice from a subsample of snails ($N = 29$ or $N = 30$ depending on the parameter) to estimate repeatability (R) of the measurements. Repeatability describes the proportion of variance in a character occurring among rather than within individuals. We calculated it from variance components derived from an ANOVA where individual was used as a factor (Krebs 1989). Repeatability of all parameters was high (hemocyte concentration: $R = 0.992$, $F_{29,30} = 62.773$, $P < 0.001$; PO-like activity: $R = 0.884$, $F_{29,30} = 3.712$, $P < 0.001$; antibacterial activity: $R = 0.931$, $F_{28,29} = 6.716$, $P < 0.001$).

Effects of temperature and exposure time on reproduction

Reproduction of snails during the experiment was measured at the end of their temperature treatment (i.e., after 1, 3, 5, 7, 9, or 11 days of exposure) using photographs taken from all the egg clutches oviposited by the snails. The images were analyzed using ImageJ (ImageJ 1.42q, Wayne Rasband; National Institute of Health, Bethesda, MD) to estimate the number of eggs in the clutches. The area containing nine to twelve eggs in each clutch was measured as well as the area of the clutch, and an estimate of the total number of eggs in the clutch was calculated. The number of eggs in the clutches oviposited by each snail was then summed up to get a measure of its reproductive output.

Statistical analyses

To examine the effects of temperature and exposure time on snails' immune function, variation in snails' immune defense traits was first analyzed using a multivariate analysis of variance (MANOVA, with Pillai's trace test statistic for unequal sample sizes). In the analysis, hemocyte concentration (ln transformed), PO-like activity (ln transformed), and antibacterial activity of snail hemolymph were used as response variables. In the analysis, a model with temperature (15 and 25°C) and exposure time (1, 3, 5, 7, 9, and 11 days) as fixed factors was used. As the MANOVA indicated an effect of temperature on immune function (see Results), separate ANOVAs using a similar model as above were conducted for different immune parameters to investigate whether their responses to temperature differed. When statistically significant interactions between temperature and exposure time (indicating dependence of the effect of temperature on the length of exposure) were observed in ANOVAs, differences between temperature treatments at each different exposure times were examined with planned contrasts to identify which time points were responsible for the significant interaction term.

The effect of temperature on snails' growth was examined using an analysis of covariance (ANCOVA) with snails' shell length at the end of the experiment as a response variable, temperature and exposure time as fixed factors, and snails' shell length at the beginning of the experiment as a covariate.

To estimate the effect of temperature on snails' reproduction, the variation in the proportion of snails that oviposited eggs in the experiment was first analyzed using a generalized linear model where the reproductive status of the snails (oviposited eggs, did not oviposit eggs) was used as a binomial response variable with logit link function. In this model, temperature and exposure time were used as fixed factors. In addition, as temperature affected snails' growth and thus size at the end of the experiment (see Results), and the capability to reproduce can be size dependent, the proportion of snails that reproduced during the study was also analyzed using a separate model in which snails' shell length at the end of the experiment was included as a covariate. This model reveals the size-independent effect of examined factors on snails' reproduction and estimates whether temperature affects reproduction directly or indirectly (i.e., via larger size). After that, the variation in the number of oviposited eggs was analyzed using only those snails that reproduced during the experiment. The total number of eggs oviposited (square root transformed) by the snails was used as a response variable in an ANOVA with a similar model as above. Because of a significant temperature by exposure time interaction in this model (see Results), differences in the total number of oviposited eggs between temperature treatments at different exposure times were tested using planned contrasts. Furthermore, the data were analyzed separately for different temperature treatments to estimate the effect of exposure time on reproduction at each temperature in detail. In these analyses, ANOVAs with exposure time as a fixed factor fulfilled with repeated contrasts that compared egg numbers between consecutive exposure time treatments were used. These contrasts reveal changes in the cumulative number of oviposited eggs over the course of a heat wave. Analyses comparing only differences between temperature treatments at each exposure time are not able to estimate that as only the total number of eggs oviposited during the experiment was measured, and snails may reproduce intermittently during the experiment. Additionally, an ANCOVA using snails' shell length at the end of the experiment as a covariate was performed using the full model to estimate size-independent effect of examined factors on snails' reproduction as above.

To examine whether immune traits, growth, and reproduction traded off with each other at individual level

and/or relationships among them depended on experimental treatments, similar models as above were conducted adding one trait at a time as a covariate (including main effects and factor by covariate interactions). No negative relationships among the traits or interactions among factors and covariates were detected (data not shown).

The assumptions of all the above analyses were fulfilled, and they were performed using IBM SPSS Statistics, version 19.0 software (IBM Corp., Armonk, NY).

Results

Effects of temperature and exposure time on immune traits

Although the immune function of the snails exposed to 25°C was lower than that of the snails exposed to 15°C (main effect of temperature, MANOVA: Pillai's trace = 0.095, $F_{3,298} = 10.460$, $P < 0.001$), exposure time modified the difference between temperature treatments (temperature × exposure time interaction, MANOVA: Pillai's trace = 0.142, $F_{15,900} = 2.973$, $P < 0.001$). The response to temperature treatment over time was not the same for all the measured immune parameters (Table 1, Fig. 1). When exposed to 25°C, hemocyte concentration of snail hemolymph was reduced in individuals exposed for 7 days (planned contrast: contrast estimate = 0.191, $P = 0.010$; Fig. 1A), but was at the same level as in individuals maintained at 15°C in the subsequent exposure time treatments (planned contrasts: |contrast estimate| ≤0.111, $P \geq 0.127$ for both; Fig. 1A). PO-like activity was reduced consistently in individuals exposed to high temperature for 7 days or longer (planned contrasts: contrast estimate ≥ 0.086, $P \leq 0.005$ for all; Fig. 1B). Antibacterial activity of snail hemolymph was not affected by temperature (Table 1, Fig. 1C).

Table 1. Analyses of variance (ANOVAs) for the immune parameters (hemocyte concentration, phenoloxidase [PO]-like activity, antibacterial activity of snail hemolymph) by water temperature (15, 25°C) and exposure time (1, 3, 5, 7, 9, and 11 days).

Source	df	MS	*F*	*P*
Hemocyte concentration				
Temperature (*T*)	1	0.034	0.462	0.497
Exposure time (*E*)	5	0.289	3.968	0.002
T×*E*	5	0.295	4.044	0.001
Error	300	0.073		
PO-like activity				
Temperature (*T*)	1	0.106	9.92	0.002
Exposure time (*E*)	5	0.054	5.052	<0.001
T×*E*	5	0.077	7.223	<0.001
Error	300	0.011		
Antibacterial activity				
Temperature (*T*)	1	118.868	2.795	0.096
Exposure time (*E*)	5	684.786	16.102	<0.001
T×*E*	5	10.599	0.249	0.940
Error	300	42.527		

MS, mean squares.

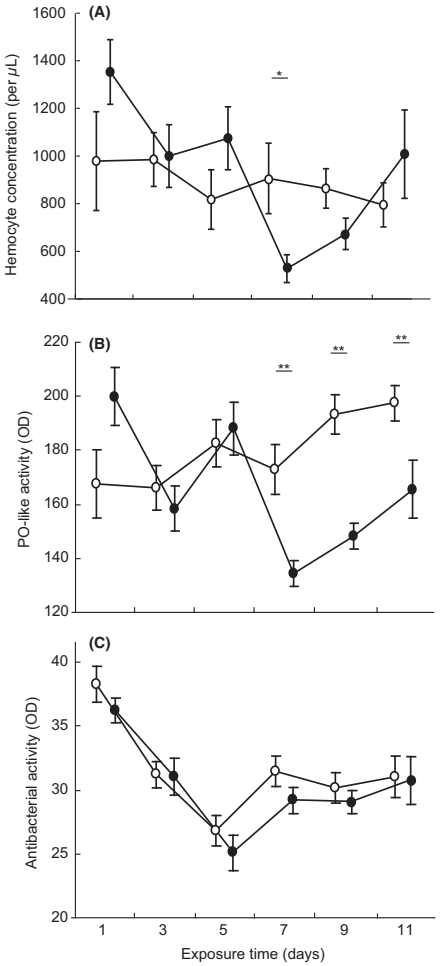

Figure 1. (A) Hemocyte concentration (mean ± SE), (B) phenoloxidase (PO)-like activity (mean ± SE), and (C) antibacterial activity (mean ± SE) of hemolymph for snails exposed to 15°C (open circles) and 25°C (filled circles) for different times (1–11 days).
*significance level < 0.05, **significance level < 0.01.

Effects of temperature and exposure time on growth and reproduction

The snails exposed to 25°C grew larger than snails exposed to 15°C (Table 2, Fig. 2). The proportion of snails that oviposited eggs during the experiment was also higher at 25°C (Table 3, Fig. 3A). This effect did not depend on their final size (generalized linear model: Wald $\chi^2 = 0.775$, $P = 0.379$). For the snails that reproduced, the total number of eggs that were oviposited was higher at 25°C (Table 4, Fig. 3B). The effect of temperature on egg production, however, depended on exposure time (Table 4) so that the number of oviposited eggs by snails exposed to 25°C for 5, 7, 9, and 11 days was higher than the number of eggs oviposited at 15°C (planned contrasts: contrast estimate ≤ -3.518, $P \leq 0.004$ for all). When the total number of eggs was analyzed separately for each temperature treatment, a statistically significant difference in the cumulative number of oviposited eggs among

Table 2. Analysis of covariance (ANCOVA) for snails' shell length at the end of the experiment by water temperature (15, 25°C) and exposure time (1, 3, 5, 7, 9, and 11 days) with initial size as a covariate.

Source	df	MS	*F*	*P*
Temperature (*T*)	1	4.881	16.603	<0.001
Exposure time (*E*)	5	6.830	23.232	<0.001
Initial size	1	2432.713	8274.971	<0.001
T×*E*	5	0.622	2.117	0.063
Error	299	0.294		

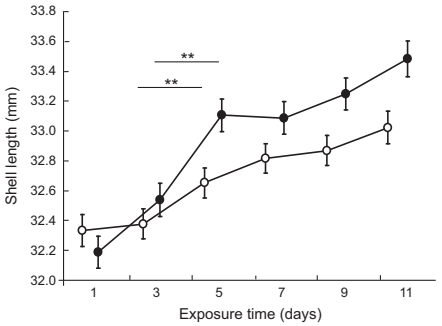

Figure 2. Final size adjusted for initial size (mean ± SE) for snails exposed to 15°C (open circles) and 25°C (filled circles) for different times (1–11 days).
**significance level < 0.01

Table 3. Generalized linear model for reproductive status (oviposited eggs, did not oviposit eggs) of snails by water temperature (15, 25°C) and exposure time (1, 3, 5, 7, 9, and 11 days).

	Wald χ^2	df	*P*
Temperature (*T*)	3.965	1	0.046
Exposure time (*E*)	46.613	5	<0.001
T×*E*	1.802	5	0.876

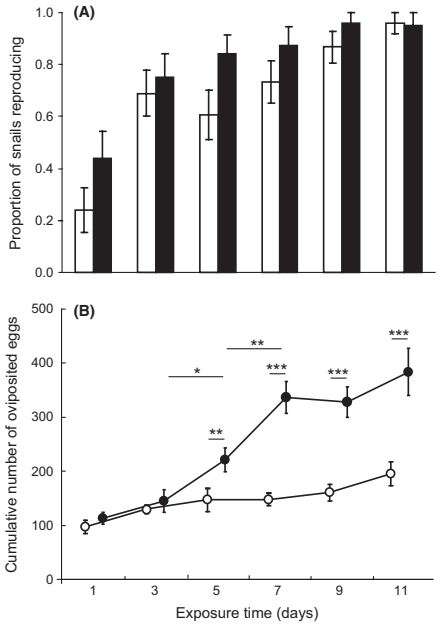

Figure 3. (A) Proportion of snails that oviposited eggs and (B) the number of oviposited eggs for those snails that reproduced in the experiment (mean ± SE) when exposed to 15°C (open bars/circles) and 25°C (filled bars/circles) for different times (1–11 days). *significance level < 0.05, **significance level <0.01, ***significance level < 0.001.

Table 4. Analysis of variance (ANOVA) for the total number of oviposited eggs by water temperature (15, 25°C) and exposure time (1, 3, 5, 7, 9, and 11 days).

Source	df	MS	*F*	*P*
Temperature (*T*)	1	801.273	59.528	<0.001
Exposure time (*E*)	5	168.752	12.537	<0.001
T×*E*	5	54.898	4.078	0.001
Error	218	13.460		

different exposure times was only found in snails exposed to 25°C (ANOVAs: 25°C: $F_{5,110} = 12.621$, $P < 0.001$; 15°C: $F_{5,108} = 2.130$, $P = 0.067$; Fig. 3B). In this treatment, the number of eggs oviposited by the snails differed significantly between individuals exposed for 3 days and for 5 days, and between individuals exposed for 5 days and for 7 days (repeated contrast: contrast estimate ≤ -3.007, $P \leq 0.025$ for both, Fig. 3B). This indicates that the observed increase in egg numbers at 25°C compared with 15°C (see above) was due to higher reproductive rate in short-term exposures to high temperature and that long-term exposures did not lead to a further increase in oviposition. Shell length at the end of the experiment affected the number of eggs oviposited (ANCOVA: $F_{1,217} = 17.629$, $P < 0.001$). However, the effects of other factors remained qualitatively similar in this model when compared with the model without a covariate (Table S1).

Discussion

Extreme weather conditions such as summer heat waves are predicted to become more frequent in the future due to global climate change (Meehl and Tebaldi 2004). This is suggested to have considerable impacts on natural populations and communities, for example, by changing species' ranges, phenology, and interactions among them (see Parmesan 2006; Walther 2010 for review). Here, we show that exposure to high ambient temperature has direct implications for immune defense and life history traits in a freshwater snail *L. stagnalis* and that the overall phenotypic response depends on the duration of the experimental heat wave. When exposing snails either to 15°C or 25°C for a maximum of 11 days, we found that short experimental heat waves led to a plastic response that did not alter snails' immune function but increased their growth and reproduction. When the experimental heat wave was longer than a week, the negative effects of high temperature became pronounced. Snails' immune function decreased considerably, and reproductive output did not show a further increase compared with short experimental heat waves. This suggests that snails were not able to maintain high performance under increased temperature over a long time period. Thus, only long-term exposures to high temperature can reduce snails' immune defense and predispose them to parasite infections.

The observed response in short experimental heat waves that increased the relative expression of growth and reproduction but did not alter immune defense traits could be explained by adaptive phenotypic plasticity where the optimal resource allocation among traits changed in order to maximize fitness under altered environmental conditions. According to life history theory, optimal resource allocation among traits that are important for self-maintenance (e.g., immune defense) and reproduction depends on the reproductive value of an individual (Fisher 1930). The reproductive value is composed of the current reproductive value and the age-specific shape of the residual reproductive value function and captures the trade-off in resource allocation between maintenance and current reproduction (Fisher 1930; Williams 1966). As exposure to high temperature can increase organisms' metabolic rate and this way fasten aging (Philipp and Abele 2010), this can lead to a shift in the reproductive value, and hence alter allocation of resources among traits. However, contrary to our expectation, immune defense was not reduced at high temperature to maintain other traits, but reproduction and growth were increased while immune function was not affected. This is likely to be due to general physiological changes caused by high temperature that increase performance. According to the van 't Hoff and Bredig (1900) principle, an increase of 10°C should enhance the speed of biochemical processes two to fourfold. In fact, the effect of temperature on metabolic rate is demonstrated in many ectothermic species (e.g., Iguchi and Ikeda 1995; Person-Le Ruyet et al. 2004), and this can also explain the increased performance of snails exposed to short experimental heat waves. However, assuming that high temperature was generally beneficial for the physiology, one would also expect an increase in the level of constitutive immune defense traits (Angilletta 2009; Karl et al. 2011). One would especially expect this because higher temperature enhances the growth of potentially pathogenic microorganisms in water (White et al. 1991) that may further activate immune function.

A potential explanation for the negative effects of long experimental heat waves is that decrease in snails' performance reflects the delayed physiological costs of their high growth and reproduction that is induced by short-term response to increased temperature. In our experiment, these costs are unlikely to be due to reduced physiological condition of snails as they were all fed ad libitum, and both lipid and glycogen content of their foot tissue did not differ between the treatment groups (see supplement). Therefore, downregulation of the immune system and reproduction may follow from the demand that physiologically challenging conditions pose on necessary repair and protection mechanisms of tissues when exposure to high temperature continues for a longer period of time. For example, investment in production of heat-shock proteins (Hsp70) is known to increase with increasing temperature (Hofmann and Somero 1995). Additionally, exposure to high temperature may lead to a higher requirement for micronutrients (e.g., zinc, copper, vitamins) (see Askew 1995 for review, Chen et al. 2003) as they are involved in response and tolerance

mechanisms (Hänsch and Mendel 2009). Hence, the risk of running out of essential micronutrients can increase with the time organisms are exposed to challenging conditions, and the resulting under-supply may limit performance. In this study, both of these potential explanations are possible and cannot be distinguished. Furthermore, they are probably not mutually exclusive. As both explanations are resource dependent, long experimental heat waves would probably have had a stronger effect if snails were kept under restricted food supply (Moret and Schmid-Hempel 2000; Adamo et al. 2012).

Interestingly, temperature did not have a similar effect on all measured immune parameters in our experiment. While hemocyte concentration and PO-like activity of snail hemolymph were reduced at high temperature, antibacterial activity did not differ between the treatments. The reason for this is unknown, but it is possible that different immune traits respond differently to environmental variation as they are produced through different pathways. For example, the immune traits examined in this study are also known to differ in their responses to starvation of snails (Seppälä and Jokela 2010). It is also possible that antibacterial activity is a more important component of snails' immune defense than hemocyte concentration and PO-like activity and therefore maintained at the same level also under harsh environmental conditions. This could be because different immune parameters have various functions in the snails' immune system. Hemocytes are involved in phagocytosis and encapsulation (as well as in many other functions of the hemolymph) (Sminia et al. 1973; Sminia 1981; van der Knaap et al. 1993), PO is a part of oxidative defenses (Söderhäll and Cerenius 1998; Loker 2010), and antibacterial proteins are specialized against microbial infections (Hancock and Scott 2000). Thus, because of the increased growth rate of microorganisms under high temperatures (White et al. 1991), snails may aim to maintain antibacterial activity of their hemolymph at a high level although reducing other immune traits. Furthermore, it is possible that different immune traits have different thermal optima and therefore vary in their responses to high temperature (Murdock et al. 2012). However, the exact reasons for the differences in the responses of different immune parameters to temperature variation as well as its consequences for snails' defense against different types of parasites remain to be investigated.

Potential ecological consequences entailing these results are manifold. First, the change in reproductive pattern may change snails' population dynamics as well as interactions within and among species (e.g., competition, predation; see Walther 2010 for review). However, the effects of the enhanced reproduction in short-term exposures to high temperature have to be interpreted with caution as we do not know whether temperature affects the quality of the produced offspring (Gilchrist and Huey 2001). Second, snails may become more susceptible to parasites, which can have severe negative impacts on host populations as has been shown in other systems (Massad and Forattini 1998; Pounds et al. 2006). Furthermore, if the above responses of snails to high temperature show genetic variation, this could even alter host–parasite coevolution by changing the effects of host and parasite genetics in determining the outcomes of host–parasite interactions as described in other systems (Blanford et al. 2003; Thomas and Blanford 2003; Mitchell et al. 2005).

In conclusion, short experimental heat waves led to a plastic response that changed the relative expression of fitness related traits by increasing growth and reproduction. This, however, did not impair snails' immune defense. When exposed to long experimental heat waves, negative effects on snails' performance became pronounced as snails' immune function was reduced and reproductive output did not further increase compared with short-term exposures. The fact that snails could not maintain high performance over prolonged experimental heat waves possibly indicates longer term costs of investment in physiological repair and protection mechanisms. Thus, our study demonstrates that more frequent and longer heat waves, as predicted under global climate change scenarios, can alter the expression of life history traits of organisms in natural populations and subject them to parasites by changing the levels of host defenses.

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Conflict of Interest

None declared.

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Supporting Information

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

Appendix S1. Measurement and analyses of energy reserves
Table S1. Analysis of covariance (ANCOVA) for the total number of oviposited eggs.

APPENDIX S1

Measurement and analyses of energy reserves

Lipid and glycogen content of tissue reflects the energy reserves the snails have available for physiological functions. From a subsample of 81 snails (four to eight individuals per temperature by exposure time combination) tissue samples were taken from the foot (Josse and Van Elk 1986) to measure the lipid and glycogen contents. Samples were snap frozen in liquid nitrogen, and stored at -80°C for later analysis. To measure the lipid content, tissue samples were dried at 60°C for 48 h. After that, their dry mass was measured to the nearest 0.01 mg (balance: Sartorius R200D, precision 0.01 mg). Then each sample was placed in a glass vial and covered with ether. After 24 h, samples were washed with fresh ether to remove all lipids, dried at 60°C for 48 h, and weighted again. The amount of lipids in the tissue was calculated by subtracting the weight after lipids were removed from the weight before lipids were removed.

Glycogen content was measured from 10 mg of tissue. Samples were homogenized in 200 µl of cold water. Homogenates were boiled for 5 min to inactivate enzymes. Boiled samples were centrifuged at 13000 rpm for 5 min. Glycogen analyses were done using the EnzyChrom Glycogen Assay Kit (BioAssay Systems, Hayward, CA, USA). OD was measured on 10 µl and glycogen concentrations were determined using a standard curve.

Variation in energy reserves (lipid and glycogen content of snail feet) was analysed with a MANOVA using temperature and exposure time as fixed factors. Lipid content was arcsinesquareroot transformed and glycogen content was squareroot transformed to fulfil the assumptions of MANOVA.

Results of energy reserves

Energy reserves of the snails (i.e. lipid and glycogen content in foot tissue) were not affected by temperature (MANOVA: main effect of temperature: Pillai's trace = 0.043, $F_{2,68} = 1.509$, $p = 0.228$; temperature by exposure time interaction: Pillai's trace = 0.186, $F_{10,138} = 1.418$, $p = 0.179$). Snails' foot tissue contained on average 0.018 mg lipid/mg dry weight (SE ± 0.061) and 108.528 µg/ml glycogen (SE ± 7.652).

TABLE S2

TABLE S2 Analysis of covariance (ANCOVA) for the total number of produced eggs by water temperature (15°C, 25°C) and exposure time (1, 3, 5, 7, 9, 11 days) using final size as covariate.

source	df	MS	*F*	*p*
temperature (T)	1	1945.746	62.001	0.000
exposure time (E)	5	800.178	25.498	0.000
T × E	5	70.793	2.256	0.049
final size	1	346.268	11.034	0.001
error	299	31.382		

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II

DIRECT AND CROSS-GENERATIONAL EFFECTS OF AN EXPERIMENTAL HEAT WAVE ON EARLY LIFE STAGES IN A FRESHWATER SNAIL

by

Katja Leicht & Otto Seppälä

Manuscript

III

INFECTION SUCCESS OF *ECHINOPARYPHIUM ACONIATUM* (TREMATODA) IN ITS SNAIL HOST UNDER HIGH TEMPERATURE: ROLE OF HOST RESISTANCE

by

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RESEARCH Open Access

Infection success of *Echinoparyphium aconiatum* (Trematoda) in its snail host under high temperature: role of host resistance

Katja Leicht[1,2] and Otto Seppälä[1,3*]

Abstract

Background: Extreme weather events such as summer heat waves become more frequent owing to global climate change and are predicted to alter disease dynamics. This is because high temperatures can reduce host immune function. Predicting the impact of climate change on host-parasite interactions is, however, difficult as temperature may also affect parasite infective stages and other host characteristics determining the outcome of interaction.

Methods: Two experiments were conducted to investigate these phenomena in a *Lymnaea stagnalis–Echinoparyphium aconiatum* (Trematoda) interaction. In the first experiment, the effects of exposure of snails to experimental heat waves [maintenance at 25°C vs. 15°C (control)] with different durations (3 days, 7 days) on the infection success of parasite cercariae was examined. In the second experiment, the infection success was examined under similar conditions, while controlling for the possible temperature effects on cercariae and at least partly also for host physiological changes that take place rapidly compared to alterations in immune function (exposure to cercariae at intermediate 20°C).

Results: In the first experiment, increased infection success at 25°C was found independently of the duration of the heat wave. In the second experiment, increased infection success was found only in snails maintained at 25°C for 7 days, a treatment in which snail immune defence is known to be impaired.

Conclusions: These results suggest that the effects of host resistance in determining overall parasite infection success can be overridden by effects of temperature on parasite transmission stages and/or alterations in other host traits than immune defence.

Keywords: *Echinoparyphium aconiatum*, Global climate change, Heat wave, *Lymnaea stagnalis*, Resistance to infection, Host-parasite interaction, Experimental assessment

Background

The outcomes of host-parasite interactions and therefore disease dynamics are often affected by environmental conditions (see [1,2] for review). Especially as ambient temperature can heavily influence these dynamics [3,4]. Owing to anthropogenic climate change, understanding such effects is increasingly important as climate change leads to an increase in average air temperature and more frequent occurrence of extreme weather events such as summer heat waves [5,6]. The effect of high temperature on host-parasite interactions is often assumed to take place via reduced resistance of hosts to infections due to impaired immune function [7,8]. This is because immune defence is typically considered to be the main physiological barrier against parasites (see [9] for review).

Predicting the effects of temperature on host-parasite interactions based only on the knowledge on host immune defence can, however, be difficult. This is because parasite infection strategies as well as other host characteristics than immune function can be important in determining the outcome of interactions. For instance, temperature can directly affect parasite infective stages (e.g. survival, mobility) thus modifying their infection success [10-13]. Similarly, in ectothermic species, temperature affects host metabolism and could modify their physiological traits (see [14] for review). For example,

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metabolic products, excreted into the environment by the organisms are used for host finding by some parasite species (see for review [15-17]), and temperature could alter excretion of such chemical host cues. However, the role of the above effects is likely to be system specific [18-20]. This could cause high variation in the effects of temperature across different host-parasite interactions complicating the predictions of the effects of climate change [21]. Hence, examining the infection success of parasites under high temperature and the role of altered host defences in determining this is in high demand.

This study focused on the effect of high ambient temperature on the infection success of *Echinoparyphium aconiatum* (syn. *Pseudoechinoparyphium echinatum*; Trematoda) cercariae in its snail host *Lymnaea stagnalis*. In this system, high temperature is known to reduce several immune defence traits of snails [22,23], and thus snails can be assumed to become less resistant to infections during heat waves. This effect, however, is seen only when snails are maintained at high temperature for one week or longer [23]. The parasite *Echinoparyphium aconiatum* is one of the most common trematode species infecting *L. stagnalis* [24-26]. It has a complex life-cycle including snails as first and second intermediate hosts, and snail-eating birds as a definitive host [27]. Cercariae released from the first intermediate host use chemical cues (e.g. amino acids) to find their second intermediate host from the environment [15,18,28], which they enter through the urinary orifice and develop into encysted metacercariae mostly in the hepatopancreas.

Here, the effects of experimental heat waves on the interaction between *L. stagnalis* and *E. aconiatum* cercariae were examined in two experiments. The first experiment investigated their effects on the overall parasite infection success. The second experiment, examined infection success by controlling for the possible temperature effects on cercariae and at least partly for host physiological changes that take place rapidly compared to alterations in immune function which are slow in this system [23]. As experimental temperatures of 15°C [a common temperature in habitats of snails (A. Laurila, 2010, unpublished data; U. Tobler, 2010, unpublished data)] and 25°C [a temperature that occurs intermittently in ponds during hot summers (A. Laurila, 2010, unpublished data; U. Tobler, 2010, unpublished data)] were used. As only long-term (one week or longer) maintenance at high temperature is known to impair immune function of snails [23] the effects of both short- (3 days) and long-term (7 days) heat waves were tested. We predict overall parasite infection success to increase (experiment 1) and host resistance to infection to decrease (experiment 2) after long-term maintenance of snails at high temperatures. If temperature affects parasite transmission stages and/or other host traits than immune defence we expect host resistance to be a poor predictor for parasite infection success.

Methods

Experimental animals

Experimental snails came from a laboratory stock population (F_2 generation) originating from a pond in Zürich, Switzerland (47°22′N, 8°34′E). The population was maintained in water tanks (temperature ranging from 12°C to 20°C, but being close to 15°C for most of the year) for one year before the experiment. Five days prior to the experiment (see below), experimental snails (shell length: 27.6 - 42.6 mm) were randomly chosen from the population and placed individually in plastic cups filled with 200 ml of aged tap water at 15°C to acclimatize them to the experimental conditions. Snails were fed with spinach *ad libitum*, and water in the cups was changed every second day during this period and throughout the following experiments.

Snails releasing *E. aconiatum* cercariae for the experiment originated from ponds in Biengarten, Germany (49°39′N, 10°49′E). With using an allopatric parasite a coevolutionary history between the host and the parasite could be avoided. Snails collected from the field were brought to the laboratory and placed individually in plastic cups filled with 40 ml of water. Snails infected with *E. aconiatum* were identified by observing the morphology of released cercariae (see [25]). Infected snails were maintained in boxes, with 10 to 15 snails in each, filled with 6 l of aged tap water at 20°C (a typical temperature at the collection site at the time of sampling; own obs.) and fed with spinach *ad libitum* between collecting and use in the experiment.

Experimental design

The study was conducted in two experiments that ran simultaneously. The first experiment examined the overall effect of high temperature on the infection success of *E. aconiatum* in *L. stagnalis*. Experimental snails were maintained as described above and randomly assigned into one of the two temperature (15°C, 25°C) and maintenance time (3 days, 7 days) treatments (20 snails per treatment combination). Water in the cups was changed once more after the maintenance period and snails were exposed to parasite cercariae (see below) at their respective maintenance temperatures.

The second experiment examined the effect of high temperature on the resistance of snails to infection when controlling for the potential direct effects of temperature on parasite cercariae and partly also for host physiological changes that take place rapidly compared to alterations in immune function and could affect the outcome of interaction (see the Discussion for potential mechanisms). Experimental snails were maintained as in experiment 1.

After the maintenance period, snails were removed from their cups and transferred into new cups filled with aged tap water at 20°C (temperature at which the snails producing cercariae were maintained). Immediately after the transfer, snails were exposed to *E. aconiatum* cercariae (see below).

After the maintenance period in experimental treatments (see above), snails were exposed to freshly emerged (5–30 min old) parasite cercariae. To gain cercariae, a total of 60 infected snails were transferred into cups filled with fresh water (i.e. no previously released cercariae) and emerged cercariae were collected under a microscope. In the parasite exposures, four to five different host snails were used to gain a total of 20 cercariae that were introduced into the cup of each experimental snail. Which experimental snail received parasites from which infected snails was determined by chance. Thus, each infected snail contributed equally to the mix of cercariae used for infections, and the genetic (i.e. clonal) composition of parasites varied among exposed snail individuals. The exposure dose of 20 cercariae per snail was used as it allows examining infection success as a quantitative trait [29,30], and as parasite intensities in the wild commonly vary between 10 and 30 metacercariae per snail (own obs.). Snails were exposed to the parasites for 24 h. This exposure time is sufficient for the parasites to invade the snails and form metacercariae as they are only infective for approximately 24 h after emergence [31]. After that, snails were removed from the cups and their shell length was measured to the nearest 0.1 mm. Then, snails were removed from their shells and parasites that successfully infected the snails (i.e. encysted as metacercariae into snail tissues) were counted under a microscope.

Statistical analyses

Variation in parasite infection success was analysed using generalized linear models for both experiments. In these models, the proportion of parasites successfully infecting the snails (x/20) was used as a binomial response variable (logit link function), and maintenance temperature (15°C, 25°C) and maintenance time (3 days, 7 days) were used as fixed factors. Because of a significant interaction between the factors in the second experiment (see Results), the data were further analysed separately for different maintenance times to estimate the effect of maintenance temperature in detail. In these analyses, generalized linear models as above with maintenance temperature as a fixed factor were used. Size of snails is known to partly determine the infection success of *E. aconiatum* [29]. In this study, however, the shell length of the snails did not differ among treatment groups at the end of the experiment (Additional file 1). Furthermore, using shell length as a covariate in the above models led to significant interactions between factors and the covariate. Therefore, shell length was not included into the final models. All statistical analyses were performed using IBM SPSS Statistics Version 19.0 software (Armonk, NY: IBM Corp.).

Results

In the first experiment, the proportion of cercariae that successfully infected the snails was higher in individuals maintained and exposed to parasites at 25°C than in snails maintained and exposed at 15°C (Table 1, Figure 1). This effect was independent of maintenance time (Table 1, Figure 1).

In the second experiment where the snails were exposed to parasites at 20°C, maintenance temperature of snails before parasite exposure did not have a significant main effect on parasite infection success (Table 2, Figure 2). Its effect, however, depended on maintenance time indicated by a significant interaction between the factors (Table 2). Therefore, the effect of maintenance temperature was analysed separately for different maintenance time treatments. In the long-term treatment, higher parasite infection success was found in snails maintained at 25°C than at 15°C (generalized linear model: Wald Chi-Square = 6.736, $p = 0.009$, Figure 2). In the short-term treatment, maintenance at 25°C before parasite exposure led to fewer infections than maintenance at 15°C (generalized linear model: Wald Chi-Square = 11.182, $p = 0.001$, Figure 2).

Discussion

In this paper, we show that exposure of *L. stagnalis* snails to high temperature increased the infection success of *E. aconiatum* cercariae. This, however, was not only due to decreased host resistance. In the first experiment where the snails were exposed to cercariae at their respective maintenance temperature, the proportion of parasites that successfully infected the snails was higher at 25°C compared to 15°C both in short- and long-term treatments. This indicates that other factors than the altered immune function (see [22,23]) are important in determining infection success as immune function of *L. stagnalis* is known to be reduced only after seven days of exposure to high temperature (see [23]). In the second experiment where the resistance of snails against the infection was examined by controlling for the possible direct effects of temperature on parasite infective stages

Table 1 GLM for the proportion of *E. aconiatum* cercariae infecting *L. stagnalis* in the first experiment

	Wald chi-square	df	*p*
Temperature (T)	16.921	1	<0.001
Maintenance time (D)	0.223	1	0.637
T × D	0.715	1	0.398

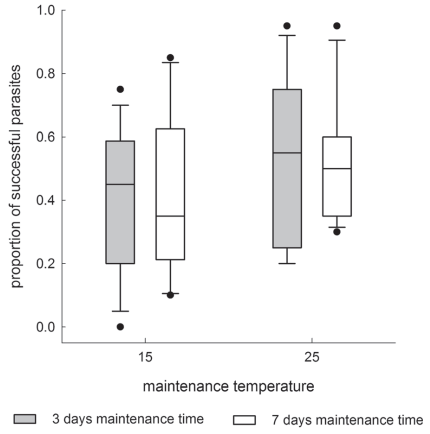

Figure 1 Infection success of *E. aconiatum* cercariae in the first experiment. Proportion of encysted metacercariae in snails maintained at different temperature treatments for three or seven days before parasite exposure (20 cercariae per snail, 11 to 20 snails per treatment group) when the snails were exposed to parasite cercariae at their respective maintenance temperature. Data are shown as box plot with median, 25th (lower box) and 75th (upper box) quartiles; whiskers represent the values within the 1.5 interquartile range (outliers are shown as ●).

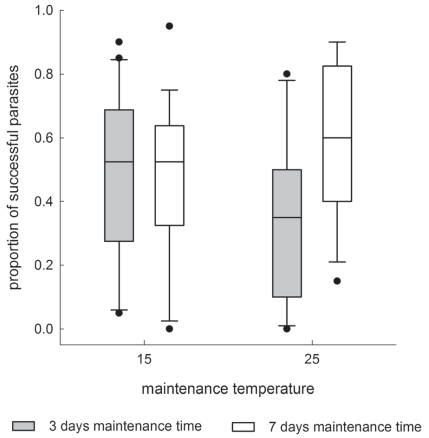

Figure 2 Infection success of *E. aconiatum* cercariae in the second experiment. Proportion of encysted metacercariae in snails maintained at different temperature treatments for three or seven days before parasite exposure (20 cercariae per snail, 11 to 20 snails per treatment group) when the snails were exposed to parasite cercariae at 20°C. Data are shown as box plot with median, 25th (lower box) and 75th (upper box) quartiles; whiskers represent the values within the 1.5 interquartile range (outliers are shown as ●).

and partly for temperature-dependent changes in host metabolic and physiologic rate (i.e. exposure at 20°C), infection success was higher only when the snails were maintained at 25°C for seven days before parasite exposure. This indicates that the resistance of snails against *E. aconiatum* is affected by temperature, and confirms that other factors need to override its effect in determining the infection success of the parasite.

The observed effect of temperature on overall parasite infection success could take place via at least two different mechanisms that may override the effect of altered host resistance. First, temperature could directly affect parasite cercariae by altering their mobility, survival, and infectivity [10,13,31,32]. For instance, an effect on the activity of cercariae can alter the contact probability between the parasite and the host leading to changed transmission efficiency [10,13,32]. Similarly, altered efficiency in the use of energy reserves can change infectivity of cercariae [10,12]. Thus, an increase in the performance of parasites at high temperature may enable cercariae to overcome host defences more easily. Second, ambient temperature may also affect other host traits than immune function, which could alter the outcome of the interaction. For instance, echinostome cercariae are able to find snails from the environment using chemo-orientation by responding to the micromolecules excreted by the snails [15,18,28]. These responses depend on the total amount of such host cues and also on their chemical composition [15,18,28]. Thus, environmental factors that alter the metabolism and physiology of snails could modify the excretion of such molecules altering exposure to parasite transmission stages. For example, physiological effects of food processing are known to predispose snails to *E. aconiatum* cercariae [29]. Similarly, ambient temperature could change the amount and composition of such chemical cues in the environment by altering snail metabolism. The relative importance of changes in host cues in the environment compared to direct temperature effects on parasite larvae might be large as chemo-orientation allows direct movement towards the host [18]. However, the actual role of these potential mechanisms remains to be investigated.

Interestingly, in the first experiment, the combined effect of reduced resistance due to long-term maintenance

Table 2 GLM for the proportion of *E. aconiatum* cercariae infecting *L. stagnalis* in the second experiment

	Wald chi-square	df	*p*
Maintenance temperature (T)	0.466	1	0.495
Maintenance time (D)	16.379	1	<0.001
T × D	17.778	1	<0.001

at high temperature and possible direct effects of temperature on parasite transmission stages and/or differences in host physiology apart from immune defence did not lead to higher infection success compared to short-term exposures. It is possible that the maximum infection success of cercariae was already reached in both treatments as not all parasites may be able to infect the snails (e.g. due to intrinsic factors of the parasite such as mortality or infectivity). The highest observed infection success of approximately 60% is lower than what has been reported for this species earlier [29,33]. The use of an allopatric host may have reduced the maximum infection success in this study (see [34] for review, [35]). Moreover, in the second experiment, the proportion of encysted parasites when the snails were maintained at high temperature for three days before parasite exposure was lower than when the snails were maintained at 15°C. This was unexpected as in our earlier study [23], none of the examined immune parameters (i.e. haemocyte concentration, phenoloxidase-like activity, and antibacterial activity of snail haemolymph) was altered by short-term maintenance at high temperature. It is important to note, however, that immune responses of snails to *E. aconiatum* are currently not well understood. Thus, it could be that some other immune traits that were not examined in our earlier study [23] determine the resistance of snails to this parasite species. Such parameters could respond differently to high temperature compared to the ones examined earlier [8], which could alter snail resistance already after short-term maintenance at high temperature. Potential candidates for such mechanisms are, for example, reactive oxygen species such as hydrogen peroxide [36] and lectins such as fibrinogen-related proteins (see [37] for review), which have been reported to be important in snail defences against trematodes in other species.

Conclusions

Exposure of snails to high temperature increased the infection success of *E. aconiatum* cercariae independently of the length of the experimental heat wave snails had experienced. However, when controlling for the effects of temperature on parasite transmission stages and potential temperature-dependent changes in host metabolism and physiology that may take place rapidly compared to changes in immune defence (e.g. excretion of chemical cues), only long-term maintenance at high temperature led to increased infection success. This suggests that such factors overrode the effects of temperature on host resistance in determining the infection success under high temperature. Our results suggest that heat waves can lead to higher parasite infection success and thus increase the probability of epidemics. However, environmental conditions can also influence at other stages of parasite life cycles. For example, the production and release of cercariae in the snail hosts is known to depend on environmental conditions in several parasite species [38-40]. Additionally, environmental stress can cause high mortality among infected hosts [41-43]. Since parasite population dynamics are determined by a balance between parasites within-host growth and reproduction, host and parasite mortality, and parasite transmission rate [44,45], all of which may vary across host and parasite species, more studies focusing on different steps of parasite life cycles are needed to assess the overall effect of climate change on disease dynamics.

Additional file

Additional file 1: Analysis of differences in the shell length of snails among treatments at the end of the experiments.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KL and OS designed and implemented the experiments, and performed the statistical analyses. KL wrote the manuscript. OS revised the manuscript. Both authors read and approved the final manuscript.

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ADDITIONAL FILE 1

Statistical analyses

To analyse differences in the shell length of snails among treatments at the end of the experiments, generalized linear models were performed for both experiments. In these models, shell length was used as a response variable (identity link function), maintenance temperature (15°C, 25°C) and maintenance time (3 days, 7 days) before parasite exposure were used as fixed factors.

Results

Shell length at the end of the experiments did not depend on experimental treatments (Tables A1, A2).

TABLE A1 GLM for the shell length of *L. stagnalis* by maintenance temperature (15°C, 25°C) and maintenance time (3 days, 7 days) before parasite exposure in the first experiment.

	df	Wald Chi-Square	*p*
maintenance temperature (T)	1	0.201	0.654
maintenance time (D)	1	3.210	0.073
T × D	1	0.136	0.712

TABLE A2 GLM for the shell length of *L stagnalis* by maintenance temperature (15°C, 25°C) and maintenance time (3 days, 7 days) before parasite exposure in the second experiment.

	df	Wald Chi-Square	*p*
maintenance temperature (T)	1	1.408	0.235
maintenance time (D)	1	0.762	0.383
T × D	1	0.010	0.921

IV

DOES INBREEDING ALTER THE EFFECTS OF HEAT WAVES IN A FRESHWATER SNAIL?

by

Katja Leicht, Jukka Jokela & Otto Seppälä

Manuscript

V

ADAPTIVE POTENTIAL UNDER CLIMATE CHANGE: FAMILY-LEVEL VARIATION IN FITNESS-RELATED TRAITS AND THEIR RESPONSES TO HEAT WAVES IN A SNAIL

by

Katja Leicht, Katri Liljeroos & Otto Seppälä

Submitted manuscript