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Heat and immunity: an experimental heat wave alters immune functions in three-spined sticklebacks (*Gasterosteus aculeatus*)

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Summary

1. Global climate change is predicted to lead to increased temperatures and more extreme climatic events. This may influence host–parasite interactions, immunity and therefore the impact of infectious diseases on ecosystems. However, little is known about the effects of rising temperatures on immune defence, in particular in ectothermic animals, where the immune system is directly exposed to external temperature change.

2. Fish are ideal models for studying the effect of temperature on immunity, because they are poikilothermic, but possess a complete vertebrate immune system with both innate and adaptive immunity. We used three-spined sticklebacks (*Gasterosteus aculeatus*) originating from a stream and a pond, whereby the latter supposedly were adapted to higher temperature variation. We studied the effect of increasing and decreasing temperatures and a simulated heat wave with subsequent recovery on body condition and immune parameters.

3. We hypothesized that the immune system might be less active at low temperatures, but will be even more suppressed at temperatures towards the upper tolerable temperature range.

4. Contrary to our expectation, we found innate and adaptive immune activity to be highest at a temperature as low as 13 °C. Exposure to a simulated heat wave induced long-lasting immune disorders, in particular in a stickleback population that might be less adapted to temperature variation in its natural environment.

5. The results show that the activity of the immune system of an ectothermic animal species is temperature dependent and suggest that heat waves associated with global warming may immunocompromise host species, thereby potentially facilitating the spread of infectious diseases.

Key-words: climate change, heat wave, heat-induced stress, immune activity, immunocompetence, parasite resistance, temperature increase

Introduction

Ecosystems are presently exposed to global warming and climate change. Current predictions state that by the end of the twenty-first century, the daily maximum temperature will increase by 1–5 °C in central Europe, which will coincide with more frequent temperature extremes, such as heat waves (IPCC 2012). In ectothermic animals, such as fish, the activity and efficiency of their immune systems is strongly temperature dependent (Ellis 2001). At the same time, metabolic rates and virulence traits of parasites are influenced and temperature is thought to be a strong modifier of host–parasite interactions (Lazzaro & Little 2009; Studer, Thieltges & Poulin 2010; Landis *et al.* 2012).

Vertebrate immunity is categorized into innate immunity, which is less specific but provides rapid protection and acquired immunity, which reacts slowly but provides high specificity and memory (Koellner *et al.* 2002). Invading pathogens are at first confronted with factors (proteins/cells) of the innate immune system, as a first line of

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defense. If pathogens survive (evade) the attack by innate immunity, acquired immunity is activated to produce highly specific antibodies to facilitate elimination of the invaders. However, ectothermic animals are generally adapted to a certain temperature range, and temperature extremes will cause stress, which may lead to decreased immune functions (Cheng *et al*. 2003; Dove *et al*. 2005; Roth, Kurtz & Reusch 2010; Seppaelae & Jokela 2011).

With the present study, we ask how the immune system of three-spined sticklebacks, a fish species that is used as an ecological model organism, is influenced by temperature. Fish are ideal organisms with which to study the effect of temperature on immunity: on the one hand, fish are ectothermic, such that the immune system is directly exposed to changes in external temperature, in contrast to, for example, mammals or birds, which keep their body temperature largely constant. On the other hand, bony fish possess a complete vertebrate immune system that resembles the immune system of mammals. The immune activity of bony fish changes with ambient temperature within the adaptive temperature range of the species (Ellis 2001; Bowden 2008; Fig. 1). At low temperatures (<4 °C), fish immunity is suppressed, which might be attributed to low or absent infectivity of pathogens at these temperatures. When temperature increases, innate immune activity increases first; this is probably sufficient to maintain immunocompetence at relatively low temperatures (Ellis 2001). At the species-specific intermediate temperature range, both innate immunity and acquired immunity are fully active and provide optimal immunocompetence. At high temperatures, above the species-specific optimum, innate and acquired immune functions tend to be suppressed (Fig. 1). Fish immunity is fully active in the intermediate temperature range of a species, but suppressed at low and presumably at high temperatures.

The majority of studies on the effects of temperature on fish immunity have been performed with fish bred in aquaculture, without an ecological perspective. The three-spined stickleback (*Gasterosteus aculeatus*) is now established as a model for investigations on temperature effects in a wider ecological context, as it is distributed across different fresh- and saltwater habitats and its adaptive temperature range depends on the thermal regime of the respective habitat of origin. It was observed that growth, survival and reproductive rates of sticklebacks are temperature dependent (summarized in Fig. 2). Continuous maintenance of sticklebacks 4 °C above the ambient temperature in warmed mesocosms resulted in a 60% reduction in stickleback population biomass (Moran *et al*. 2010).

The first evidence that stickleback immunocompetence is involved in such losses in biomass was provided by a field enclosure experiment during the extreme heat wave in Europe in summer 2003. High mortality (> 75%) was observed in sticklebacks exposed to their natural parasites in the field enclosures at water temperatures up to 24·3 °C (Wegner *et al*. 2008). However, detailed experimental studies directly addressing the effect of temperature on the immune system of this important ecological model organism are currently lacking.

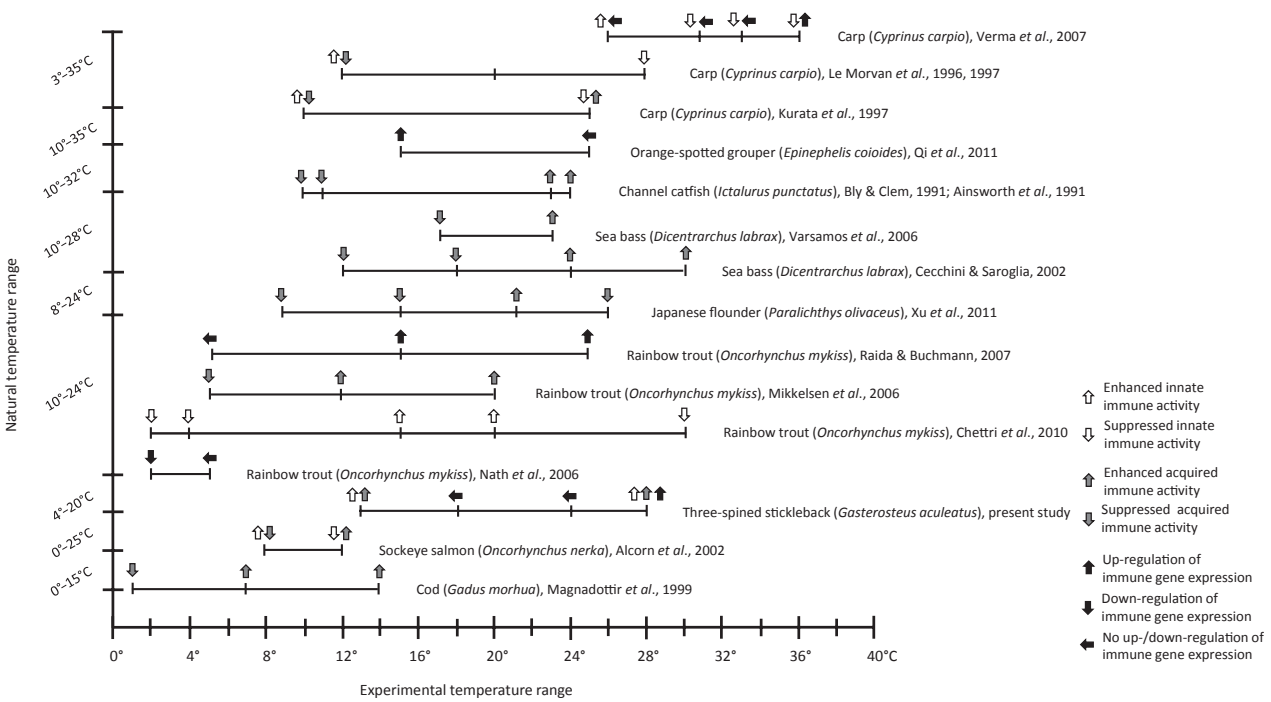

Fig. 1. Overview of temperature effects on immune activity in several fish species. Horizontal bars on the *x*-axis show the range of temperatures examined in previous studies. Arrows denote the conclusions of respective studies at the given temperature. The *y*-axis represents the natural temperature range of the respective fish species.

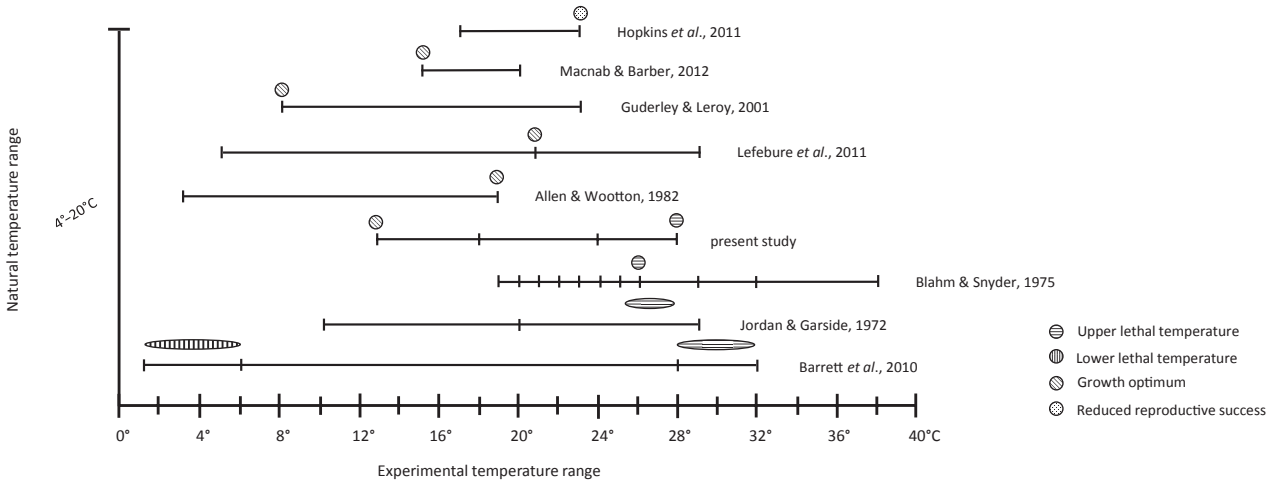

Fig. 2. Overview of temperature effects on stickleback growth, survival and reproduction. Horizontal bars on the *x*-axis show the range of temperatures examined in previous studies. Circles denote the conclusions of respective studies at the given temperature. The *y*-axis represents the natural temperature range of sticklebacks.

Sticklebacks are typically present in waters with temperatures ranging from 4 to 20 °C (Allen & Wootton 1982; Fig. 2). Water temperatures around 18 °C are common during average summers in central Europe and were used as control temperature in the present study. We used 13 °C as the lower experimental temperature, as it lies well within the natural temperature range of sticklebacks. To investigate stickleback immune activity at their upper thermal range, we used 28 °C, which sticklebacks are able to tolerate after appropriate acclimation (Jordan & Garside 1972; Barrett *et al.* 2010; Fig. 2) and which is in line with the ≥ 24 °C estimate, which has been predicted for global climate change scenarios (EEA 2012; IPCC 2012).

In the present study, we addressed the hypothesis that increasing environmental temperatures could immunocompromise three-spined sticklebacks. We further hypothesize that populations of sticklebacks may differ, such that 'cold-adapted' stickleback populations are more susceptible to high temperatures than 'warm-adapted' sticklebacks. Therefore, we compared sticklebacks (F1) originating from a stream, with relatively low summer temperatures and a pond, with higher summer temperatures.

We studied the effect of temperature on body condition parameters and a number of immune parameters that are representative of innate or acquired immune functions. We hypothesized that the activity of innate and acquired immunity would be highest at the intermediate (18 °C) temperature and would decrease upon challenge with lower and higher temperatures. The organs used for immunological parameters were (i) spleens, with the main function to trap and eliminate debris of self and non-self cells produced during cellular turnover and immune responses (innate and acquired) from the blood and (ii) head kidneys, the major lymphatic organ of fish, in which immune cells (of the innate and the acquired system) are produced and matured according to demands in the periphery.

Isolated head kidney leukocytes were analysed for their respiratory burst activity as a parameter of innate immunity. Granulocytes, as representatives of innate immunity, and lymphocytes, the main effectors of acquired immunity, were quantified, and granulocyte-to-lymphocyte (G/L) ratios were calculated to estimate the activity of the innate versus the acquired system. Cellular innate activity was assessed by measurements of proliferation of monocytes and acquired activity by the proliferation of lymphocytes.

We further hypothesized that expression profiles of immune relevant genes would be influenced by temperature change and analysed gene expression profiles from spleens in one of the experiments. Selected candidate genes were representatives of innate and acquired immune functions, as well as immune regulatory genes. We had similar expectations for the expression of functional immune genes as for the cellular responses, namely that the highest expression rates might occur at intermediate (18 °C) temperatures and that gene expression would be reduced at low and high temperatures. For the expression of cytokines, the mediators of the immune system, we expected increased expression rates at high temperatures, as the crosstalk between the neuroendocrine and the immune system might be influenced by a stress response to a thermal challenge.

As responses to temperature may change with time, sticklebacks were generally sampled prior to, immediately after and 2 weeks after temperature change, and also 2 weeks after recovery at control temperature in a heat wave experiment.

The experiments were (i) *temperature shift* experiment, where we investigated the effect of a temperature change from a supposed optimal temperature (18 °C) to a suboptimal (13 °C) and above optimum (24 °C) temperature; (ii) *heat wave* experiment, where we investigated how a

simulated heat wave (temperature increase up to 28 °C, followed by recovery at 18 °C) might alter the immune activity of sticklebacks originating from a stream and a pond population (the latter supposed to be more adapted to temperature variation); and (iii) *temperature kinetics* experiment, where we tested whether a higher speed of temperature increase (up to 4 °C per day) would have a stronger effect on immune function.

Materials and methods

STICKLEBACK HUSBANDRY

Sticklebacks were maintained in aquaria blocks with recirculated tap water and temperature control. Each block consisted of six small aquaria (14 L), one large aquarium (80 L) and a separate tank for water filtration and temperature regulation (Vewa Tech®, Hamm, Germany). Experimental sticklebacks were laboratory-bred offspring (F1) of sticklebacks caught in spring 2009 from a stream [Meckelbach (MB); 51°55′55·66″N, 7°34′36·42″E] and two ponds [small pond (SP); 51°56′00·15″N, 7°34′33·39″E and large pond (LP); 51°55′59·81″N, 7°34′30·28″E] in the area of Münster, North-West Germany. Offspring from the MB stream sticklebacks, with relatively low temperatures (July 2009, daily min/max 15·8/18·3 °C), and the LP pond population, with higher temperatures (20·9/24·5 °C) in the same season, were used in the *heat wave* and the *temperature kinetics* experiment. The pond was constructed in 1995 in a sports park and discharges into the adjacent stream via a cascade. Based on neutral genetic markers, the two populations are genetically distinct, and despite the relatively short separation time, differential adaptations might have occurred (Scharsack *et al.* 2012). Prior to experiments, sticklebacks were kept at 18 °C and 15/9 h light/dark cycle in family groups and fed *ad libitum* daily, initially with live *Artemia salina* naupliae and, once they had reached 2 months, with frozen mosquito larvae.

EXPERIMENTS

For experiments, sticklebacks from at least 3 (3–9) families per habitat origin were used (Fig. 3). As effects of spine clipping were not detectable in the (first) *temperature shift* experiment (data not shown), sticklebacks were marked by clipping their dorsal spines so that fish from different origins could be kept in the same aquarium. After clipping, sticklebacks were transferred to experimental groups in the 14-L aquaria of respective blocks and maintained for 2 weeks at 18 °C. In each block, one aquarium was stocked with reserve sticklebacks of the same origin. In cases of mortality ($n = 30$) in the experimental aquaria, reserve sticklebacks were transferred to the experimental tanks to maintain the same numbers of fish throughout the experiment.

Temperature treatments were assigned randomly to each of two replicate blocks. Per sampling time point, all sticklebacks from one aquarium per block were used. In each experiment, sticklebacks were sampled before the temperature change (day 0), immediately after temperature change and 2 weeks thereafter (Fig. 3a–c). In the *heat wave* experiment (Fig. 3b), sticklebacks were also sampled 2 weeks after temperatures were returned to 18 °C. In all experiments, sticklebacks maintained constantly at 18 °C served as controls.

SAMPLING OF STICKLEBACKS

Sticklebacks were euthanized with an overdose of MS222 (tricaine methanesulfate, Sigma-Aldrich, Germany). Standard length (from the snout to the base of the caudal fin, to the nearest 1 mm) and wet weight (to the nearest 1 mg) were measured. The head kidneys were dissected out and transferred to cell strainers (40 μm; BD Falcon, USA) in petri dishes with 1 mL RPMI 1640 medium (PAA, Germany) with 10% distilled water (R-90) on ice. Livers, spleens and gonads were removed and weighed to the nearest 0·1 mg. Spleens were transferred to RNAlater (Quiagen, Hilden, Germany), kept overnight at 4 °C, removed from RNAlater and then stored at −80 °C. Suspensions of stickleback head kidney

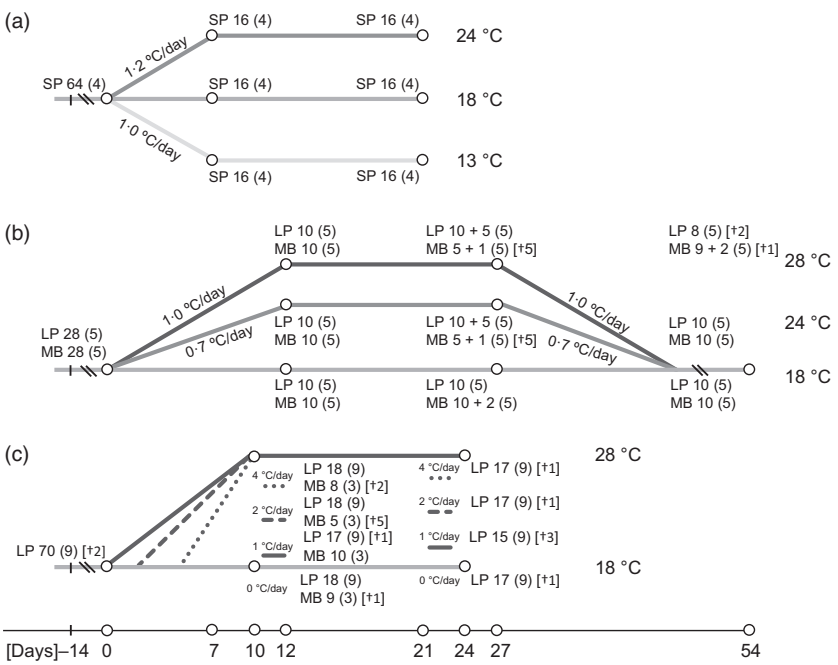

Fig. 3. Experimental set-up of the *temperature shift* (Fig. 3a), the *heat wave* (Fig. 3b) and the *temperature kinetics* (Fig. 3c) experiment. Sticklebacks were spine-clipped, distributed to experimental tanks (d-14), maintained at 18 °C and sampled before, immediately after and 2 weeks after temperature change (open circles). In the *heat wave* experiment, an additional sampling was done after temperatures were returned to 18 °C. Stickleback populations: small pond (SP), large pond (LP), Meckelbach stream (MB); numbers = n sticklebacks sampled per time point and treatment, additive numbers = n additional reserve sticklebacks sampled, numbers in parentheses = n stickleback families, numbers in parentheses = n dead sticklebacks.

leucocytes (HKL) were prepared by forcing the head kidney tissues through the cell strainers. Isolated HKL were washed twice (5 min 600 × g) with R-90 and resuspended in a final volume of 500 μL (Scharsack *et al.* 2004). Body condition index (BCI) = 100 × [weight fish/length fish $^{(\text{slope of regression})}$] and splenosomatic index (SSI) = 100 × (weight spleen/weight fish) were calculated.

FLOW CYTOMETRIC ANALYSIS

Differential cell counts of HKL were performed with a flow cytometer (BD FACS Canto II; Becton Dickinson, USA). Data were analysed with the FACS DIVA v 6.1.2 software (Becton Dickinson, USA). Cellular debris (low scatter characteristics) and aggregated cells (high scatter characteristics) were subtracted from further evaluation. Leukocytes of the innate (granulocytes and monocytes) and the acquired immune system (lymphocytes) were counted in separate gates. For the adjustment of cell numbers for the respiratory burst assay, total cell numbers were determined in freshly isolated HKL with the standard cell dilution assay (Pechhold, Pohl & Kabelitz 1994) modified by Scharsack *et al.* (2004): washed HKL (10 μL) were measured with propidium iodide (2 mg l^{-1}, Sigma-Aldrich) and standard particles (4 μm green fluorescent microspheres; Polysciences, Eppelheim, Germany). Standard particles (green fluorescence positive) were discriminated from viable HKL (propidium iodide negative, green fluorescence negative). Absolute numbers of HKL per well were calculated as follows: N [vital cells] = events [vital cells] × number [standard particles] added/events [standard particles]. To estimate the relative activity of the innate versus the adaptive immune system, suspensions of freshly isolated HKL were used to measure granulocyte (FSC/SSC high)-to-lymphocyte (FSC/SSC low) ratios (G/L ratio) in individual HKL isolates (Scharsack *et al.* 2004).

To determine leukocyte proliferation, the proportion of lymphocytes (acquired immunity) and monocytes (innate immunity) in the S and $G_{2\text{-}M}$ phase of the cell cycle were evaluated after DNA labelling with propidium iodide. Isolated HKL ($1{\cdot}25 \times 10^6$ mL^{-1}) were fixed with ethanol (200 μL cell suspension in 800 μL ice cold ethanol 99%, Roth, Germany) and stored at 4 °C. Before measurement, cells were centrifuged (10 min 600 × g) and the supernatant removed. Cells were resuspended with 150 μL/well FACS Flow (Becton Dickinson) and RNAse (0·3 mg L^{-1}, Roth, Germany) and incubated at room temperature for 10 min first and subsequently with propidium iodide (5 mg L^{-1}, Sigma-Aldrich). Individual samples were measured until 20 000 events had accumulated in the single cell gate. Doublet cells were discriminated from single cells as described by Wersto *et al.* (2001). Lymphocytes and monocytes were identified according to their characteristic FSC/SSC profile. Frequencies of lymphocytes and monocytes in $G_{0\text{-}1}$, S and $G_{2\text{-}M}$ phase were acquired by DNA content analysis of red fluorescence intensity (propidium iodide labelling) of single cells from the lymphocyte and monocyte gate.

RESPIRATORY BURST ACTIVITY

During the respiratory burst, oxygen radicals are produced by leukocytes of the innate system (granulocytes/monocytes) to destroy pathogens. Respiratory burst activity among freshly isolated head kidney leukocytes (HKL) was quantified in a lucigenin-enhanced chemiluminescence assay modified after Scott & Klesius (1981), as described by Kurtz *et al.* (2004). In white 96-well flat-bottomed microtitre plates, 80 μL of freshly isolated HKL (1×10^5 well) was added to 20 μL lucigenin solution (2·5 g L^{-1} PBS; Sigma-Aldrich) and 80 μL R-90. Plates were incubated for 30 min in the dark at 18 °C to allow uptake of lucigenin. Phagocytosis and respiratory burst were initiated by the addition of 20 μL zymosan suspension (7·5 g L^{-1} PBS; Sigma-Aldrich). Luminescence generated by the interaction of oxygen radicals and lucigenin was recorded for 3 h at 18 °C using a Tecan infinite 200 luminescence reader (Tecan, Crailsheim, Germany) using a 3-s integration time at intervals of 5 min. We tested effects of incubation temperatures (13, 18 and 24 °C) of the chemiluminescence assay with HKL from sticklebacks adapted to 13, 18 and 24 °C in a fully crossed design (Dittmar, J. unpublished data). Incubation at 24 °C increased the respiratory burst activity of unstimulated HKL from all *in vivo* temperature groups to the levels of zymosan-stimulated HKL. This effect was stronger in HKL from sticklebacks at 13 and 18 °C, which lost responsiveness to zymosan stimulation at 24 °C. The respiratory burst activity of unstimulated HKL incubated at 13 and 18 °C was lower than at 24 °C and was significantly triggered in zymosan-stimulated HKL from all *in vivo* temperature groups, but most prominently at 18 °C incubation temperature with HKL from the 13 °C *in vivo* group. We therefore decided to measure the respiratory burst activity throughout the present study at 18 °C. The respiratory burst activity of individual HKL samples was expressed as relative light units (RLU), and results were calculated as area under the curve (RLU area) with the MAGELLAN v 6 software (Tecan).

QUANTITATIVE REAL-TIME PCR

Sticklebacks from the *heat wave* experiment sampled 2 weeks after temperature change were used for gene expression profiling in spleen tissue by means of quantitative real-time PCR. Target genes were chosen as representatives of acquired humoral immunity [immunoglobulin M (IgM)], acquired cellular immunity [major histocompatibility complex class II beta (MHC IIB)], genes of innate humoral immunity [complement factor 3 (C3)], innate cellular immunity [manganese superoxide dismutase (MnSOD)] and immune regulation, namely the cytokines interleukin 1 beta (IL-1β), tumour necrosis factor alpha (TNFα) and the transforming growth factor beta (TGFβ). The heat shock proteins 70 and 90 (HSP70 and HSP90) were used as positive controls, as alteration of expression profiles of the genes by temperature (stress) is expected. Ubiquitin (ubc) and ribosomal protein L13a (rpl13a) were used as reference genes. Respective primer sequences are given in Table 1. Spleens were homogenized using a Retsch MM 301 mixer mill (2 × 2 min 25 Hz). Total RNA was extracted using the InviTrap® RNA Tissue HTS 96 Kit/C (Stratec Molecular, Berlin, Germany) according to the manufacturer's protocol. Extracted RNA was controlled for integrity on a 1·5% agarose gel and the OD 260/280 values were measured using NanoPhotometer™ Pearl (Implen GmbH, Munich, Germany) to ensure RNA purity (OD ≥ 1·8). Reverse transcription into complementary DNA (cDNA) was performed using the Fermentas First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Schwerte, Germany).

Gene expression was measured using the ABsolute™ QPCR SYBR® Green ROX Mix (ABgene, Hamburg, Germany) in a LightCycler® 480 Real-Time PCR System (Roche). The cycling parameters were as follows: 15-min enzyme activation at 95 °C followed by 40 cycles of 15-s denaturation at 95 °C and 1-min extension at 60 °C. Results were analysed using the LIGHTCYCLER®

Table 1. Primers for gene expression profiling

Gene	5′–3′ sequence	Reference
MHC IIB	fw: AACTCCACTGAGCTGAAGGACAT re: CAGTGAAGCCGACAWACTTCC	Wegner, Reusch & Kalbe (2003)
IgM	fw: AAGGCAGGAGAATGAAACCTTGG re: CCGAGTGAGCAGACAGGACTGG	Hibbeler, S. (unpublished data)
C3	fw: ATCCTTTTGACACTCTGCGTCTG re: AACCATCAAAGAAGGAAGCAAGG	Hibbeler, S. (unpublished data)
MnSOD	fw: ATGTGACCGCTCAGATTGC re: CTGGTTAGCACAAGCAGCTACG	Erin, N. (unpublished data)
HSP70	fw: GAGCCATGACCAAGGACAAT re: ATGTCCTCCTTGCTCAAACG	Present study
HSP 90	fw: CAAGGTCATTCGCAAGAACA re: GTGTCATCGCCAGACTGAGA	Present study
TGF ß	fw: TGTCCTTCGACGTCACTGAG re: GGTGGTTGCTTTGTCCTCAT	Present study
TNFα	fw: TACGTTGAGGCAAATCAGCA re: AGGACGACTGGCTGTAGACG	Present study
IL 1ß	fw: GCAGTTCGCCGCCACATCTCCAGATCAG re: CGCAGGGTGCAGGTACGCCGACATGGTC	Krause (2011)
ubc	fw: AGACGGGCATAGCACTTGC re: CAGGACAAGGAAGGCACC	Hibbeler, Scharsack & Becker (2008)
rpl13a	fw: CACCTTGGTCAACTTGAACAGTG re: TCCCTCCGCCCTACGAC	Hibbeler, Scharsack & Becker (2008)

480 v 1.5 software (Roche, Germany). The relative quantities (RQ) of target transcripts in individual samples were assessed according to Pfaffl 2001. The results are given as mean ± S.E. of RQ values [RQ = $(E_{target})^{\wedge}\Delta CT_{target}$(control-sample)/$(E_{ref})^{\wedge}\Delta CT_{ref}$(control-sample)].

STATISTICAL ANALYSIS

Statistical analyses were performed with the SPSS v 20 software (IBM, USA). Data were tested for normality with the Kolmogorov–Smirnov test and by visual examination of histograms. Data were Box–Cox-transformed if normality was not achieved. Homogeneity of variance was tested with the Levene's test.

Effects of main factors (time, temperature, gender, population) and second- and third-degree interactions on response variables were analysed with generalized linear models (GzLM) using full factorial designs. Aquarium block (nested within temperature) was added to the model to test for block effects. Models were fitted by stepwise reduction of non-significant ($P > 0{\cdot}05$) terms. Multiple comparisons were analysed with post hoc tests (least significant differences, LSD).

One-way ANOVAS were used to test whether blocks assigned to the different temperature treatments differed from each other before temperature change (day 0).

Independent sample t-tests were performed to identify significant differences within treatment groups before (day 0) and after temperature change. T-tests were Bonferroni-corrected for multiple testing ($P = 0{\cdot}025$).

For survival analysis of stream versus pond sticklebacks, life tables were produced to examine the distribution of time-to-event variables (time points). In addition, the distribution was compared by levels of a factor variable (temperature treatment). Wilcoxon test was used to compare survival distribution between groups.

Results

TEMPERATURE SHIFT

In this experiment, we tested how temperature decrease and increase influence body condition and immunity of sticklebacks. Laboratory-bred offspring from a population inhabiting a small pond (SP) were exposed to temperature change from 18° to 13° and from 18 to 24 °C (Figs 3a and 4). Effects of the temperature treatment are summarized in Table S1 (Supporting information).

During the experiment, the body condition index (BCI) of sticklebacks at 13 °C increased, but decreased at 24 °C (Fig. 4a), presumably a consequence of increased metabolic rates at 24 °C. Correspondingly, sticklebacks were longer at 13 °C (40 ± 3·47 mm, mean length ± S.D.) than at 18 °C (39 ± 2·69 mm) and 24 °C (38 ± 3·10 mm).

According to our hypothesis, immune activity was altered immediately after temperature change. A prominent drop in the respiratory burst activity of stickleback head kidney leucocytes (HKL), a parameter of innate immunity, was detected immediately after the temperature increase from 18 to 24 °C (Fig. 4c). Correspondingly, we observed a trend for lower monocyte proliferation at 24 °C (Fig. 4e). This suggests that immediately after temperature increase, cellular innate immunity (respiratory burst, monocyte proliferation) is down-regulated. Our assumption that innate and acquired immunity would be suppressed at low temperatures was not supported. After 2 weeks, parameters of cellular innate immunity, the respiratory burst activity and the monocyte proliferation were higher at 13 °C compared with 18 and 24 °C

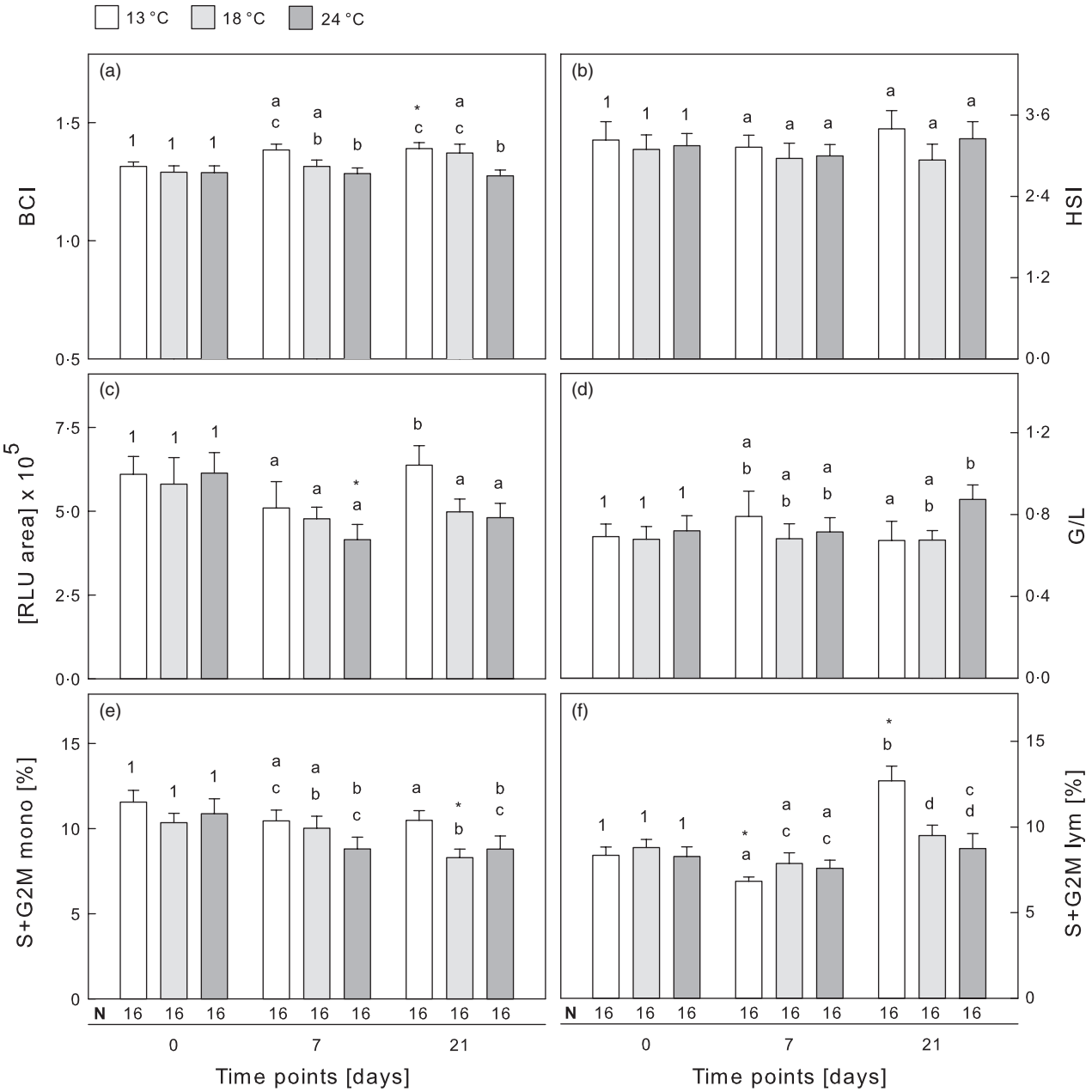

Fig. 4. Temperature shift. Mean (± S.D.) of body condition parameters (a) body condition index (BCI), (b) splenosomatic index (SSI) and immune parameters of head kidney leukocytes (c) respiratory burst activity, (d) granulocytes to lymphocytes ratio, (e) monocyte and (f) lymphocyte proliferation before and after temperature change from 18 to 13 °C and to 24 °C, respectively. Different numbers above bars indicate significant differences between treatments (day 0; one-way ANOVA, $P < 0{\cdot}05$). Different letters indicate significant differences between treatments on days 7 and 21 (GzLM post hoc, $P < 0{\cdot}05$). Asterisks indicate significant differences within treatments over time (days 0–7, days 0–21; independent samples t-test, Bonferroni-corrected, $P < 0{\cdot}025$). Note that on day 0 all groups were at 18 °C, but are given in grey scale corresponding to the respective future treatment (13, 18 and 24 °C).

(Fig. 4c,e). The representative parameter of acquired immunity, the lymphocyte proliferation, was slightly decreased immediately after temperature change from 18 to 13 °C, but was significantly increased at 13 °C compared with 18° and 24 °C two weeks after temperature change (Fig. 4f). Thus, stickleback immune activity seems to be optimal rather at 13° and not, as we expected, at 18 °C. Also, we did not find evidence to support the hypothesis that acquired immune activity would be down-regulated at high temperature, as lymphocyte proliferation did not differ between 18 and 24 °C.

HEAT WAVE

In this experiment, laboratory-bred offspring from a large pond (LP) and a stream (MB) population were exposed to temperature increase from 18 to 24 °C and from 18 to 28 °C, with a subsequent decrease back to 18 °C (Figs 3b

and 5). Effects of the temperature treatments are summarized in Tables S2 and S3 (Supporting information).

While about 50% of the stream sticklebacks died after 2 weeks of exposure to 24 and 28 °C, none of the pond sticklebacks died, indicating that stream sticklebacks are more susceptible to warming. Pairwise comparisons revealed that survival curves of stream sticklebacks were significantly different from pond sticklebacks at 24 and 28 °C (survival analysis: Wilcoxon = 8·124, d.f. = 1, $P < 0{\cdot}05$ for both 24 and 28 °C; Fig. 3b).

During the *heat wave* experiment, the body condition index (BCI) of sticklebacks from both populations increased at 18 and 24 °C, but not at 28 °C (Fig. 5a). Even 2 weeks after the heat wave, the BCI of pond and stream sticklebacks after the 28 °C treatment remained slightly below the BCI of the 18 and 24 °C sticklebacks, suggesting that both populations were affected by the heat wave (Fig. 5a). Also, the splenosomatic index (SSI) responded to the 28 °C treatment and was increased 2 weeks after the temperature increase. This effect was more prominent in stream sticklebacks (Fig. 5b).

The experiment revealed changes of innate immune activity immediately after temperature increase in either population. Again, the prediction that innate and acquired immune activity would be down-regulated at 24 °C was not supported, as differences between 18 and 24 °C in innate and acquired immune activity and immune gene expression were not detected in either population. Interestingly, immune activity was not suppressed at 28 °C as we had expected; by contrast, stream sticklebacks showed highly increased innate (Fig. 5c–e) and acquired (Fig. 5d,f) immune activity at 28 °C, whereas pond sticklebacks only showed slightly enhanced innate immune activity (Fig. 5c, e). In particular, the respiratory burst activity of stream sticklebacks was prominently increased after 2 weeks at 28 °C (Fig. 5c). Correspondingly, stream sticklebacks tended to have a higher expression of the radical scavenger manganese superoxidase dismutase (MnSOD) (Fig. 6g) and a higher granulocyte-to-lymphocyte (G/L) ratio after 2 weeks at 28 °C (Fig. 5d). This indicates that in stream sticklebacks, granulocytes were activated by the (stressfully) high temperature and that radical scavengers were produced, to limit self-damage by overshooting respiratory burst activity of these cells. The proliferation of monocytes, the precursors of granulocytes, increased immediately after temperature increased to 28 °C and remained high in both populations during the heat wave (Fig. 5e), suggesting that mobilization of monocytes (activation of cellular innate immunity) is a general feature of a sticklebacks' response to high temperatures.

Lymphocyte proliferation was low during the heat wave, except for stream sticklebacks at 28 °C (Fig. 5f). Correspondingly, stream sticklebacks at 28 °C had higher expression of the acquired immune genes IgM and MHC IIB than pond sticklebacks (Fig. 6a,b).

Contrary to our expectation that immune activity would return to baseline level after recovery from the 28 °C heat wave, the parameters of cellular innate immunity and monocyte proliferation remained increased in both populations and the respiratory burst activity remained increased in stream sticklebacks (Fig. 5c,e). The parameters of acquired immunity and lymphocyte proliferation as well were unexpectedly high 2 weeks after the heat wave, with the highest values recorded in stream and pond sticklebacks after the 28 °C heat wave (Fig. 5f). This indicates that sticklebacks need a relatively long time (>2 weeks) to readjust the activity of their immune system after a heat wave. Furthermore, temperature decrease from high to moderate seems to induce an up-regulation of acquired immune activity.

The immune regulatory genes interleukin 1 beta (IL-1β, Fig. 6c), tumour necrosis factor alpha (TNFα, Fig. 6f) and transforming growth factor beta (TGF β, Fig. 6i) tended to be higher expressed at high temperatures, supporting our assumption that cytokine expression would be up-regulated with increasing temperature. That also the expression of heat shock proteins would be up-regulated with increasing temperature is only partly supported by our findings. The expression of HSP90 tended to increase with temperature in both populations (Fig. 6h), whereas the expression of HSP70 was down-regulated in stream sticklebacks at 28 °C (Fig. 6e).

TEMPERATURE KINETICS

Inthis experiment, we tested whether the speed of temperature increase influences stickleback body condition and immune parameters. Experimental sticklebacks were again offspring from the large pond (LP) and the stream (MB) population. They were exposed to temperature increase from 18 to 28 °C at rates of 0, 1, 2 and 4 °C per day (Figs 3c and 7). While pond sticklebacks were sampled at all time points, stream sticklebacks were sampled only immediately after the temperature change on day 10 due to limited numbers of sticklebacks available from that origin. Based on the *heat wave* experiment, we expected that stream stickleback survival would be lower, especially after a steep temperature increase (4 °C per day). Here, we focussed on immediate effects of temperature increase at different rates on immune activity and survival rates. Effects of the temperature treatments are summarized in Table S4 (Supporting information).

As in the *heat wave* experiment, stream sticklebacks had higher mortalities due to temperature increase than pond sticklebacks and therefore were more susceptible to faster temperature increase. Pairwise comparisons revealed that survival curves of stream sticklebacks were significantly different from pond sticklebacks after temperature increase of 2 and 4 °C per day (survival analysis: Wilcoxon = 19·138, d.f. = 1, $P < 0{\cdot}05$ for 2 °C per day and Wilcoxon = 3·542, d.f. = 1, $P < 0{\cdot}05$ for 4 °C per day; Fig. 3c). The body condition index (BCI) tended to decrease at 28 °C, while the splenosomatic index (SSI) increased 2 weeks after the temperature increase (Fig. 7a,

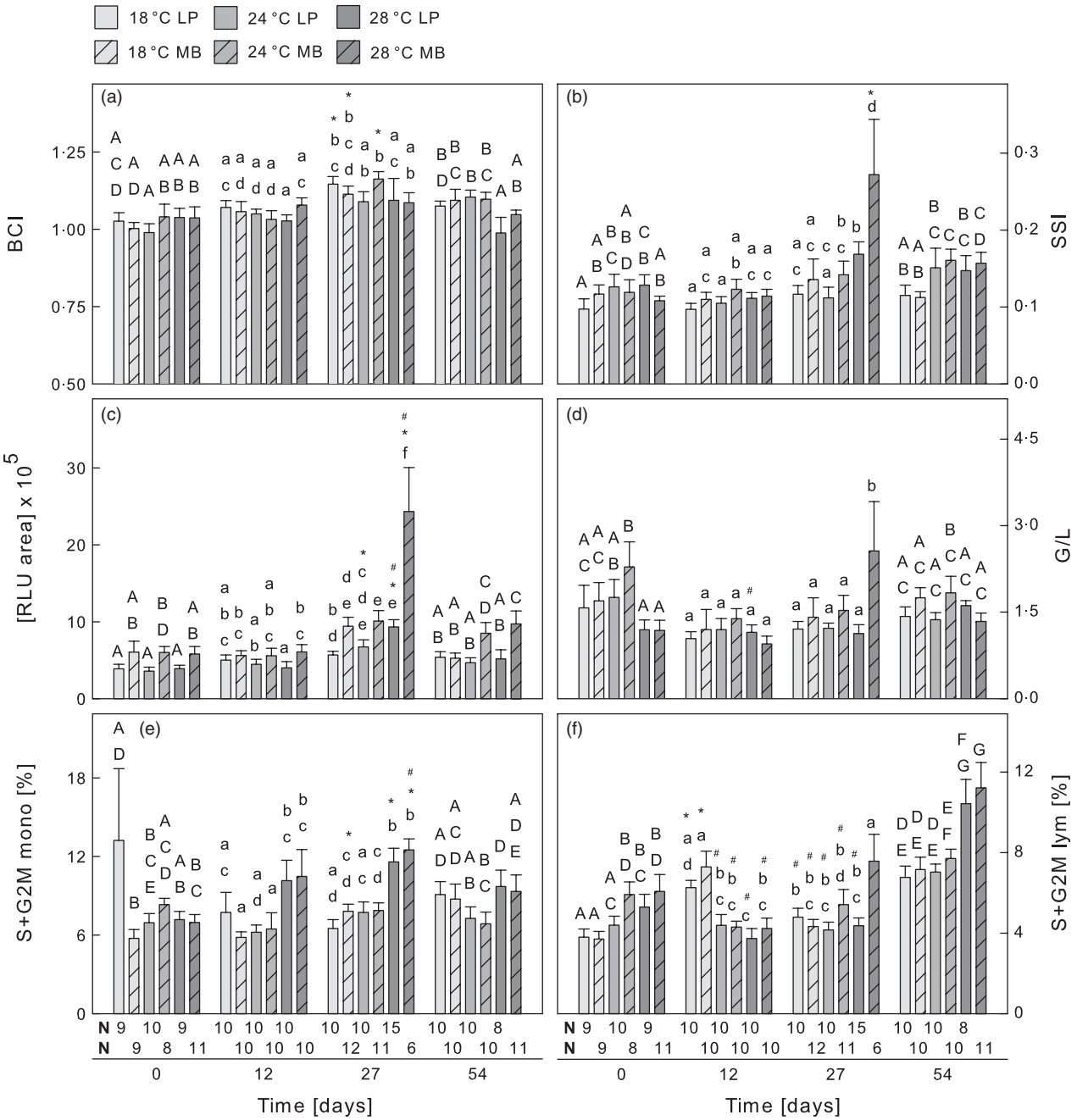

Fig. 5. Heat wave. Mean (± S.D.) of body condition parameters (a) body condition index (BCI), (b) splenosomatic index (SSI) and immune parameters of head kidney leukocytes (c) respiratory burst activity, (d) granulocytes-to-lymphocytes ratio, (e) monocyte and (f) lymphocyte proliferation before, during and after a heat wave from 18 to 24 °C and to 28 °C, respectively, in a pond and a stream stickleback population. Open bar: large pond (LP) and dashed bar: stream (MB) population, different capitals: significant difference between treatments on days 0 and 54 (GLM post hoc, $P < 0{\cdot}05$), different lower cases: significant difference between treatments on days 12 and 27 (GzLM post hoc, $P < 0{\cdot}05$), * and #: significant difference within treatments over time (days 0–12, days 0–27 and days 54–12, days 54–27; independent samples t-test, Bonferroni-corrected, $P < 0{\cdot}025$). Note that on days 0 and 54, all groups were at 18 °C, but are given in grey scale corresponding to the respective future, respectively past treatment (18, 24 and 28 °C).

b). However, effects of the speed of temperature increase on BCI and SSI were not detected.

Again, there was no evidence to support the hypothesis that innate and acquired immune activity would be suppressed at 28 °C, but we observed responses of innate and, unexpectedly, also acquired immune activity, immediately after temperature change in pond sticklebacks. Immediately after the temperature increase from 18 to 28 °C, we observed a drop in the respiratory burst activity, in the monocyte proliferation and also in the granulocyte-to-lymphocyte (G/L) ratio, which was more prominent with faster temperature increase (Fig. 7c–e). In contrast to monocyte proliferation (Fig. 7e), lymphocyte proliferation increased with faster temperature increase

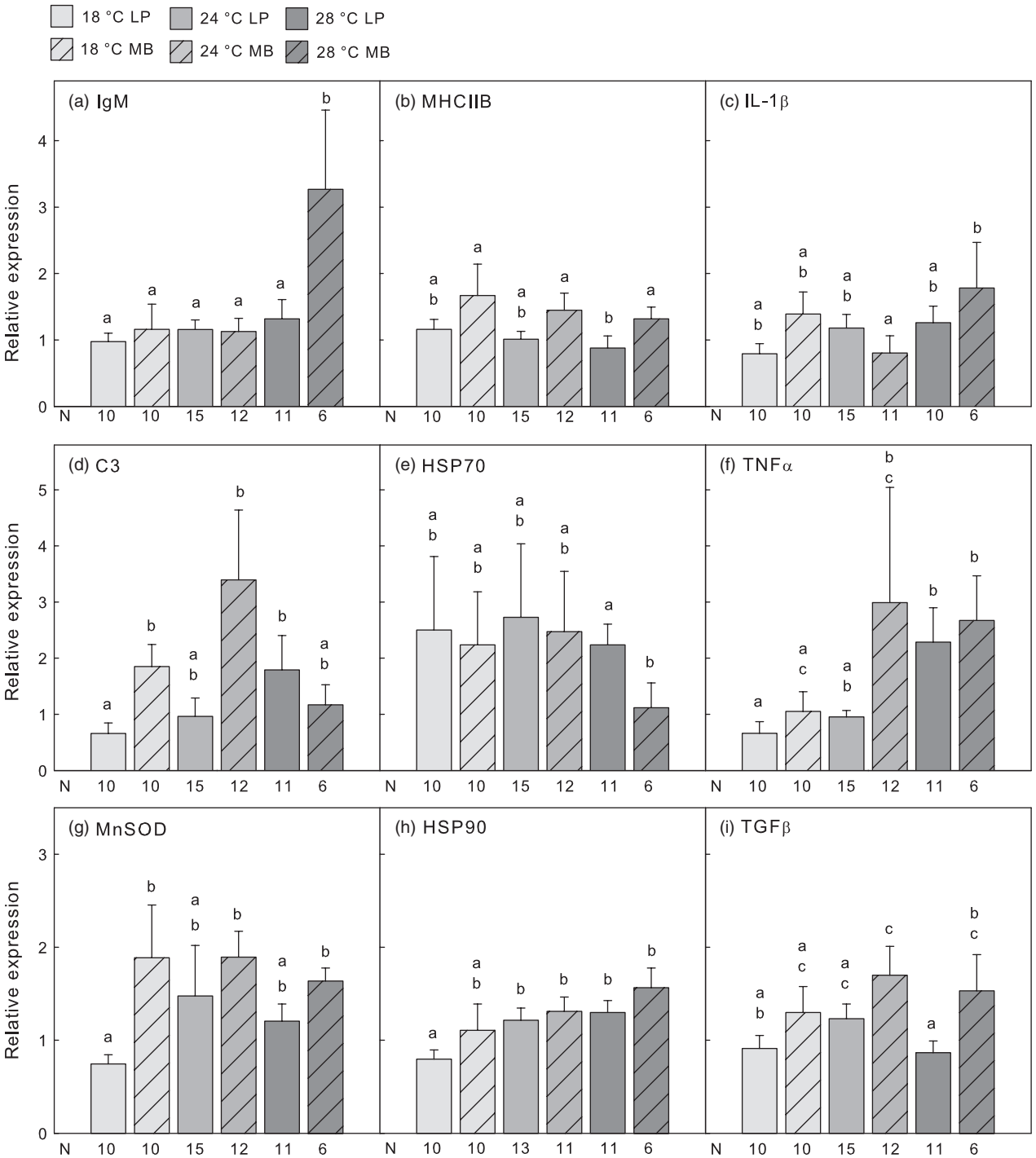

Fig. 6. Gene expression profiles during a heat wave. Mean (± S.D.) of relative quantities of target gene transcripts (a) immunoglobulin M (IgM), (b) major histocompatibility complex class II beta (MHC IIB), (c) interleukin 1 beta (IL 1β), (d) complement factor 3 (C3), (e) heat shock protein 70 (HSP70), (f) tumour necrosis factor alpha (TNFα), (g) manganese superoxide dismutase (MnSOD), (h) heat shock protein 90 (HSP90) and (i) transforming growth factor beta (TGFβ) in spleens of a pond and a stream stickleback population, 2 weeks after temperature increase (day 27, Fig. 3b). Open bar: large pond (LP) and dashed bar: stream (MB) population, different lower cases: significant difference between treatments and stickleback populations (GzLM post hoc, $P < 0{\cdot}05$).

(Fig. 7f). Two weeks later, monocyte proliferation in pond sticklebacks (LP) was increased compared to controls and lymphocyte proliferation was suppressed (Fig. 7e,f). Again, the prediction that stream and pond sticklebacks are differentially affected by high temperature was supported. Stream sticklebacks initially showed higher respiratory burst activity, which decreased with faster temperature increase, but lower monocyte proliferation than pond sticklebacks (Fig. 7c,e). Stream sticklebacks also had higher G/L ratios than pond sticklebacks, but these did not respond significantly to the speed of temperature increase (Fig. 7d).

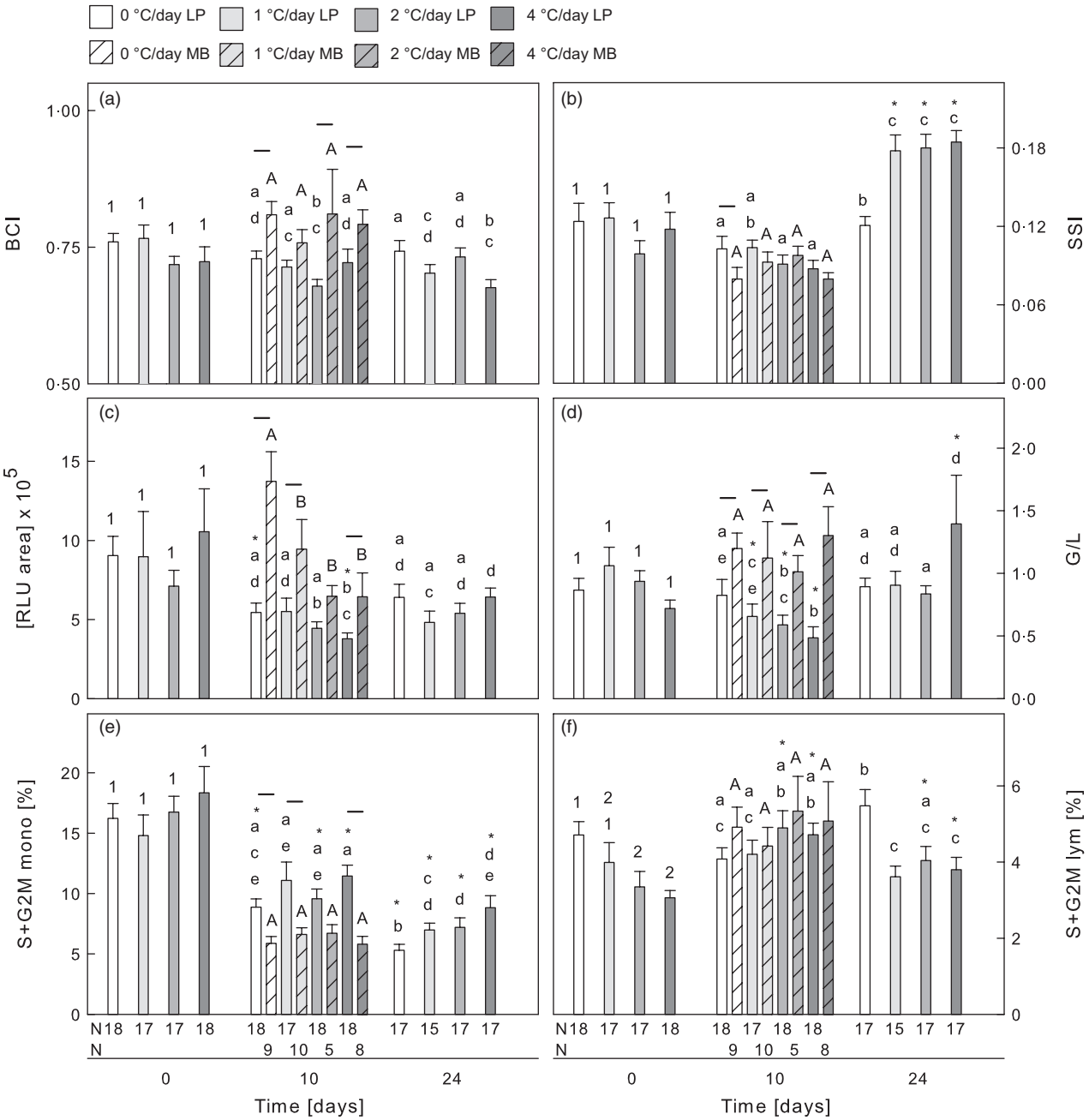

Fig. 7. Temperature kinetics. Mean (± S.D.) of body condition parameters (a) body condition index (BCI), (b) splenosomatic index (SSI) and immune parameters of head kidney leukocytes (c) respiratory burst activity, (d) granulocytes to lymphocytes ratio, (e) monocyte and (f) lymphocyte proliferation of a pond and stream stickleback population exposed to temperature increase of 0, 1, 2 and 4 °C per day from 18 to 28 °C. Open bar: large pond (LP) and dashed bar: stream (MB) population, different numbers above bars: significant differences between temperature treatments (day 0; one-way ANOVA, $P < 0{\cdot}05$), different capitals: significant difference between treatments of the stream population at day 10 (GzLM post hoc, $P < 0{\cdot}05$), horizontal lines: significant difference between the stickleback populations within the same treatment (GzLM post hoc, $P < 0{\cdot}05$), different lower cases: significant difference between treatments of the pond population at day 10 and 24 (GzLM post hoc, $P < 0{\cdot}05$), *: significant difference between pond treatments over time (days 0–10, days 0–24; independent samples t-test, Bonferroni-corrected, $P < 0{\cdot}025$). Note that on day 0, all groups were at 18 °C, but are given in grey scale corresponding to the respective future temperature kinetic (0, 1, 2 and 4 °C per day).

Discussion

It is evident that global warming influences the dynamics of infectious diseases and host–parasite interactions, with consequent implications for ecosystem functions (Harvell *et al.* 2002; Altizer *et al.* 2006; Traill *et al.* 2010). Here, we provide experimental evidence showing that the immune system is altered by changing temperatures in an ectothermic species, the three-spined stickleback. Activity of the stickleback's innate and acquired immune system

was high at relatively low (13 °C) temperatures and decreased at higher temperatures (18–24 °C). Lowered innate immune activity at increasing temperature suggests that this first line of defense might be less efficient at increased temperatures, which may facilitate invasion of ectothermic hosts by parasites. Activity of the acquired immune system as well decreased with temperature increase, which in combination with lower innate immune activity might result in higher parasite burdens of ectothermic hosts at increasing temperatures.

The immune activity of endothermic animals is not likely to be directly affected by moderate temperature increases, because body temperatures are maintained at a constant by thermoregulation. However, indirectly, endothermic animals might be exposed to higher infection pressure, if they are hosts of parasites with complex life cycles (many fish eating bird species), which themselves also use ectothermic animals as hosts.

Ectothermic animals, such as fish, are most likely differentially affected by temperature increase, as their adaptive range varies across species. In the common carp, acquired immunity was suppressed at 12 °C (LeMorvan, Deschaux & Troutaud 1996; Fig. 1), while in sticklebacks, (present study) high activity of acquired immunity was observed at 13 °C.

In the present study, the temperature decrease from 18 to 13 °C also promoted an increase in the body condition index (BCI). Interestingly, BCI was also higher at 15 °C than at 20 °C in sticklebacks, both infected and not infected with the tapeworm *Schistocephalus solidus* (Macnab & Barber 2012; Fig. 2); thus, also under immunological challenge, food conversion is more efficient at lower temperatures. We therefore conclude that 13 °C lies well within the immunological and physiological optimal temperature range of three-spined sticklebacks.

As we expected, 'cold-adapted' sticklebacks originating from a stream were more susceptible to the heat wave and suffered higher mortality than pond sticklebacks. In stream sticklebacks, mortality also increased with the speed of temperature increase. The surviving stream sticklebacks might represent a non-random subgroup of more temperature-resistant individuals. However, immune activity of pond sticklebacks responded less prominently, but in a similar manner, suggesting that up-regulation of immune activity during an extreme heat wave is rather a general phenomenon than based on individual heat susceptibility.

Contrary to our expectation, a heat wave up to the upper physiological temperature limit of sticklebacks (28 °C) resulted in increased immune activity. That this coincides with an up-regulation of immunocompetence seems unlikely. Increased mortalities of sticklebacks in field enclosures during the summer heat wave in 2003 coincided with increased parasite burden (Wegner *et al*. 2008), which suggests that thermal stress has rather negative effects on immunocompetence. A possible explanation for increased immune activity in the present study at 28 °C might be increased autoimmunity in response to cell and tissue damage caused by thermal stress. With the present data, we cannot rule out that high immune activity at high temperature is an adaptive response to potentially higher infection risk. However, here, the sticklebacks were maintained under pathogen-free conditions, and given that 28 °C is very close to the thermal maximum of sticklebacks, it is likely that tissue damage has occurred.

Sticklebacks from both populations responded to the heat wave with increased splenosomatic indices (SSI), respiratory burst activity and monocyte proliferation. The increase in SSI and respiratory burst was much more prominent in stream sticklebacks. The swelling of the spleens at 28 °C (increased SSI), in particular of stream sticklebacks, was presumably due to clearance of increased amounts of cellular debris due to immunological and physiological disorders during a thermal stress response. In the present study, stream sticklebacks showed extremely high respiratory burst activity during the heat wave. Such high levels of respiratory burst activity are potentially harmful for the host itself, as oxygen radicals might lead to increased oxidative stress and self-tissue damage (Kurtz *et al*. 2006, 2007).

The suggestion that increased respiratory burst activity might be a consequence of a dis-regulated immune system was also observed in a previous study, where sticklebacks with a non-optimal diversity of their MHC genes showed high respiratory burst activity when infected with the tapeworm *S. solidus* (Kurtz *et al*. 2004).

Oxidative stress may enhance heat sensitivity, as hydrogen peroxide inhibits the induction of HSP70 under heat stress and thereby blocks its protein refolding ability (Adachi *et al*. 2009). In catfish, *Horabagrus brachysoma*, levels of HSP70 decreased when exposed to temperatures that exceeded the optimum temperature for HSP70 induction (Dalvi *et al*. 2012). This corresponds with the down-regulation in the expression of HSP70 in stream sticklebacks exposed to 28 °C in the present study.

The differences in the responses of stream and pond sticklebacks to heat waves strongly suggest that sticklebacks adapt to the temperature regimes of their home habitats. This is remarkable in this particular case, as the pond was created in 1995 and was presumably colonized from the adjacent stream (Scharsack *et al*. 2012). Thus, differential temperature adaptation evolved within only 17 generations (sticklebacks in this area have one generation per year). This may have significant implications for future temperature increase, as sticklebacks seem to be able to adapt to changing temperature regimes relatively quickly.

Contrary to our expectation that immune activity would return to baseline levels after recovery from the heat wave, levels of immune activity remained increased, especially in stream sticklebacks. In another heat wave study with southern and northern populations of eelgrass (*Zostera marina*), gene expression profiles of southern genotypes returned to control values immediately after the heat wave, whereas the profiles of northern genotypes

revealed the induction of genes involved in protein degradation (Franssen *et al*. 2011). Thus, heat-susceptible populations likely need a longer time for recovery after a heat wave. If overshooting immune activity of stream sticklebacks in the present study was mainly due to immunological disorders, it is likely that heat-susceptible sticklebacks remain immune deficient relatively long (> 2 weeks) after a heat wave.

In conclusion, temperature was observed to be a strong modifier of the activity of the immune system of an ectothermic animal, which might heavily impact on the ecology of host–parasite systems. A heat wave caused thermal stress and immunological disorders, which likely immunocompromises the fish, an effect that even extends to some time after the heat wave. Sticklebacks from warmer habitats were less susceptible, which suggests that this species has some evolutionary potential for adaptation to global warming.

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Supporting Information

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

Table S1. Generalized linear model of main and interaction effects on body condition and immune activity of sticklebacks exposed to a *temperature shift* from 18 °C to 13° and to 24 °C, respectively (Fig. 3a).

Table S2. Generalized linear model of main and interaction effects on body condition and immune activity of sticklebacks (i) before and after the *heat wave* and (ii) during the *heat wave* (Fig. 3b).

Table S3. Generalized linear model of main and interaction effects on gene expression of sticklebacks during the *heat wave* (Fig. 3b).

Table S4. Generalized linear model of main and interaction effects on body condition and immune activity (*i*) of pond sticklebacks and (*ii*) of pond and stream sticklebacks exposed to different *temperature kinetics* (Fig. 3c).