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Interactions between immunocompetence, somatic condition and parasitism in the chub *Leuciscus cephalus* in early spring

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Relationships between immunocompetence, somatic condition, parasitism and water temperature in a wild population of chub *Leuciscus cephalus* were investigated. The effects of a rapid temperature increase in early spring were studied for both sexes as water temperature affects immunocompetence. Investment in gonads and activity of mucus lysozyme were negatively correlated; lysozyme activity decreased as temperature increased. No correlations were found between lysozyme activity and parasitism or intensity of infection by monogeneans, the most abundant metazoan parasite group in *L. cephalus*. There was a positive correlation, however, between respiratory burst intensity and parasitism. Indices of investment in gonads and spleen were correlated, showing that energetic reserves allowed either investment in gonads and spleen, or that spleen investment, even if often used in other studies in immunoecology, was not always a significant indicator of immunocompetence during this period. This last proposition is supported by the lack of correlation between spleen investment and other factors linked to immunocompetence.
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Key words: condition indices; environmental stress; immunoecology; lysozyme; spleen.

INTRODUCTION

According to life-history theory, the overall amount of energy available to any organism is limited (Stearns, 1976). Therefore, each organism must allocate its energy between several mandatory activities: survival, reproduction and somatic maintenance. Parasites, as described quantitatively by Crofton (1971), reduce the adaptive value (fitness) of their hosts by competing for a certain amount of this energy. They are also highly likely to trigger a host immune response, which is an important energetic cost for poikilotherms, such as fishes. Understanding the relationships between condition (*i.e.* a potential measure of vigour or general condition status), immunocompetence [defined as the ability to trigger, maintain and regulate an

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immune response following Kuby (1999)] and the effects of parasitism is a major goal of current immunoecology (Kurtz, 2007).

In this study, the potential interactions between these activities were investigated in the chub *Leuciscus cephalus* (L.) in early spring, when water temperature was increasing rapidly. As fish body temperature and metabolism are regulated by the environment, it could be hypothesized that several physiological, and especially immunological, mechanisms are affected by this increase. Innate immunity, because of its lower energetic cost, is more likely to be favoured over adaptive immunity in poikilotherms, especially at lower temperatures (Bly & Clem, 1991). Several mechanisms are involved in anti-parasite defence in fishes (Ingram, 1980; Buchmann, 1999; Watts *et al*., 2001; Buchmann & Lindenstrøm, 2002). Among them, the activation of lysozyme, the activation of the complement's alternate pathway, and the respiratory burst can be considered as the most important measures of innate immunity in fishes (Blazer, 1991; Buchmann, 1998; Jones, 2001).

Parasites induce an immune response in their hosts and modulate this response (Møller *et al*., 1998, reviewed the phenomenon for birds). Whether this modulation is caused by the direct action of the parasite on the host's immune system, or is related to a detrimental effect induced by parasites on the host's physiological status, however, is not yet known. Several studies using ectoparasitic monogeneans (mostly *Gyrodactylus* spp.) showed activation of the complement pathways, leading to the elimination of ectoparasites (Buchmann, 1998; Harris *et al*., 1998; Rubio-Godoy *et al*., 2004). Similarly, lysozyme is activated by ectoparasitic infection (Buchmann & Uldal, 1997; Buchmann & Bresciani, 1998). The respiratory burst is also activated by, and particularly efficient against, mesoparasites and endoparasites (Blazer, 1991; Mustafa *et al*., 2000). Unfortunately, reliable host–parasite systems for the study of innate immunity against metazoan parasites in fishes, in natural conditions, are lacking.

The goal of this study was to investigate the association between *L. cephalus* immunocompetence and condition status *v*. parasitic infection taking into account the potential effects of rapid increase of water temperature and sex differences on these associations. As temperature increase is known to trigger an increase in parasitic load, notably among monogeneans (Gelnar, 1987; Jansen & Bakke, 1991; Andersen & Buchmann, 1998), an increase in immune activity would be expected. As immunocompetence, condition and investment in reproduction are thought to interact, however, there is a need to consider these factors simultaneously in such studies, in order to control for confounding effects. The following set of measures of immunocompetence was chosen: blood cell composition, concentration of lysozyme in the mucus and maximum activity of the respiratory burst. Somatic condition was assessed using indices computed from the relative mass of three organs: spleen, hepatopancreas and gonads. In parallel, a complete parasitic examination was conducted, including identification to species of each parasite collected. The relative spleen mass was not used *strictu senso* as a measure of immunocompetence, but was compared with direct measures taken on the immune system.

To assess parasitic infection, a method taking into account both the number of each parasite species within one host individual and the total parasite, based on principal component analysis (PCA), is proposed. This indicator stresses that parasite community structure (abundance and species composition) is indeed more important, and more informative, than a single measure such as the parasite burden expressed

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as the total number of parasite individuals, or the overall diversity computed with more traditional indicators.

MATERIALS AND METHODS

FISH CAPTURE

Leuciscus cephalus were collected by electrofishing at the same location of the Oslava River, South Moravia (49° 12′ N; 16° 09′ E), on 3 April 2007 (water temperature: 6·1° C, $n = 18$, seven males and six females), 14 April 2007 (water temperature: 10·1° C, $n = 21$, eight males and 13 females). These two dates were selected at the beginning of the spring, when increase in temperature should be related to the start of parasite transmission and potentially activation of the immune system. The temperature increased gradually between these two dates (monitored each morning a month before and after the sampling period). Temperature was recorded at the same time of day. Immediately after capture, each fish was marked individually by cutting a small portion of the caudal fin. After blood sampling, fish were placed in plastic bags containing the original water with continuous oxygen input and transported immediately to the laboratory, where they were stocked in oxygenated ponds prior to dissection. It was assumed that neither parasite loss nor transfer occurred because of the short transport time (*c*. 1 h).

SAMPLING BLOOD AND MUCUS

Blood was taken in the 5 min following capture by caudal venipuncture (Stolen *et al*., 1993), and 500 µl from each individual was pooled with heparin (50 U ml^{-1}) and transferred to an icebox. Prior to transportation, the point of needle insertion was disinfected with 40% ethanol to minimize risk of infection. A 20 µl volume of mucus collected before dissection, by gently scraping the skin with a scalpel blade, was placed into sterile tubes.

FISH DISSECTION, PARASITE COLLECTION AND IDENTIFICATION

Fish were killed by a sharp blow on the head, opened, then the internal organs removed carefully and the sex determined. Spleen, gonads and hepatopancreas were weighed on a precision balance (±0·0001 g), after being carefully cleaned to prevent drops of saline or blood adding to the mass of these organs. Dissection was performed using the method of Ergens & Lom (1970). The external organs (skin, gills, fins and eyes) were examined for metazoan ectoparasites (Monogenea, Crustacea, Mollusca and Hirudinea) and internal organs (spleen, hepatopancreas, swimbladder, intestine and heart) for metazoan endoparasites (Trematoda, Nematoda, Cestoda and Acanthocephala). All parasites were collected and fixed: Monogenea in a mixture of glycerine-ammonium picrate, Nematoda in glycerin-ethanol and Digenea, Cestoda, Acanthocephala, Crustacea and Mollusca in 4% formalin. Endoparasites preserved in 4% formalin were subsequently stained using iron aceto-carmine (IAC; as described in Georgiev *et al*., 1986) and mounted on a slide with Canada balsam. An Olympus BX 20 light microscope equipped with phase contrast, differential interference contrast (DIC) and digital image analysis [Olympus MicroImage™ for Windows 95/98/NT 4.0 (Olympus Optical Co; www.olympus.co)] was used for parasite measurements and identification. Parasites were identified using recent identification keys and methodology (Ergens & Lom, 1970; Gusev, 1985; Scholz, 1989; Moravec, 1994, 2001). A complete list of species found during this study is given in the Appendix I.

HAEMATOLOGY

Blood smears were air-dried for 10 min and stained with May–Grünwald (10 drops per slide, 3 min), water and phosphate-buffered saline (PBS, 1:1, pH 7·1, 10 drops per slide,

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1 min), Giemsa–Romanovsky and distilled water (2:40, 10 drops per slide, 25–30 min) (Svobodová *et al*., 1991). Slides were air-dried after the staining process.

Slides were examined at ×1000 total magnification and 100 leucocytes were counted and identified on each slide. Each cell type (lymphocytes, monocytes, blast cells and several developmental stages of neutrophils: myelocytes, metamyelocytes, bands and segments) was determined according to shape (Lehmann *et al*., 1994) and its proportion in the total leukocyte population counted.

In parallel, for each fish, a mixture of 25 μl blood and 4975 μl Natt–Herick solution (Svobodová *et al*., 1986; Lusková, 1997) was prepared. This mixture was held for 10 min at 4° C, and then gently agitated. A volume of 20 μl was pipetted into a Bürker's haemocytometer, and erythrocytes and leukocytes were counted (Stolen *et al*., 1993) as soon as possible after fish capture (<4 h) to prevent cell degradation.

LYSOZYME CONCENTRATION

Lysozyme concentration was determined *in vitro* by radial diffusion in agarose. This method used *Micrococcus luteus* (CCM 169), which is the most sensitive microorganism dissolved by this enzyme. Glass plates (18·0 cm × 8·5 cm) covered by 26 ml of agarose containing *M. luteus* were used. Samples [5 μl of mucus from each fish or calibration solution, Lysozyme, E.C.3.2.1.17, (Sigma-Aldrich; www.sigmaaldrich.com)] were applied into the well cut in the agarose. Incubation was carried out in a wet box at room temperature (20° C) for 24 h. Diameters of lytic zones were recorded after 24 h. The content of lysozyme in each sample was converted according to a calibration curve into mg ml^{-1} of mucus.

RESPIRATORY BURST ACTIVITY

Two mixtures containing 25 μl of luminol (Molecular Probes; www.invitrogen.com), dissolved in borate buffer, pH 9, final concentration 10^{-3} mol l^{-1}], 25 μl of zymosan particles [Zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich), final concentration of 0·25 mg ml^{-1} reaction mixture, opsonized by incubation with serum from different *L. cephalus*] and 200 μl of ×40 diluted fish blood in Hank's balanced salt solution were prepared for each individual (Nikoskelainen *et al*., 2004). The kinetics of luminol-enhanced chemiluminescence was measured for 1 h at room temperature (20–25° C) using a luminometer (MLX Microtiter Plate Luminometer, Dynex Technologies; www dynextechnologies.com); 40 measurements were taken. The measured values included the maximal intensity of respiratory burst (in relative light units, RLU) and its total intensity defined as reaction curve area, integral (RLU s^{-1}). Values were corrected by the number of cells able to trigger oxidative burst.

CONDITION INDICES

Fish were weighed (total body mass, M_T in g) and measured (total length, L_T, in mm). For hepatopancreas, spleen and gonads, condition indices were calculated as follows: for each individual, the residuals of linear regression between $\log_{10}$ transformed values of M_T and $\log_{10}$ transformed values of organ mass, which is the observed deviance of organ mass for each individual, were compared with the expected mass of this organ at a given individual mass. Because of the existence of strong allometric relationships between organ masses and M_T in fishes, which leads non-independent measurements being compared, the ratio used was not the usually computed one (Bolger & Connolly, 1989) but rather residuals from linear regressions after $\log_{10}$ transformation.

STATISTICAL ANALYSIS

As distribution of variables was not normal (Kolmogorov–Smirnov test), differences between the two temperatures for each of them were estimated using a Mann–Whitney *U*-test. A composite value (the structure-intensity index, I_{SI}) for parasitism was created by extracting values for the first axis of PCA performed on the individual number of each parasite

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species per individual host. This indicator allowed the capture of both population abundance and species diversity, which are assumed to be more indicative than parasitic load or diversity alone. Even if this method, because it is derived from data at population level, is difficult to apply in comparative studies, it has the advantage of carrying information on the community structure, as well as, being extracted from a PCA, to compute distances in terms of parasitism between individuals. An R function to calculate this index was derived (pers. data).

To assess global trends of associations between indicators, a PCA was conducted on indices of spleen, hepatopancreas and gonad investment, number of neutrophils, lymphocytes, lysozyme activity, respiratory burst intensity and composite value for parasitism. For each temperature, correlations between variables were more thoroughly tested by a Spearman rank-correlation test. All tests were significant at $P < 0{\cdot}05$.

PCA was conducted between indicators of immunocompetence, in order to determine the interactions between them, and to assess which fraction of variance was explained with the set of measurements.

RESULTS

Mean ± S.E. L_T and M_T were 226 ± 31 mm, $125{\cdot}1 \pm 59{\cdot}8$ g for males and 253 ± 37 mm, $175{\cdot}4 \pm 87{\cdot}0$ g for females, with no significant variation in host morphometric variables between the two sampling days (females being significantly longer than males). Descriptive statistics for host morphometrics, physiology and immunocompetence are given in Table I. Data relative to parasitism are given in Table II.

The PCA showed that the first two axes accounted for most of the total variability in the data set. The first axis explained 36·53% and the second explained 19·77% of

TABLE I. Overview of physiological and immunes data collected on male (M) and female (F) *Leuciscus cephalus* at two different temperatures. Values are means ± S.E.

Sex	Males		Females	
Temperature (° C)	6·1	10·1	6·1	10·1
L_T (cm) (S,T,*,§)	$20{\cdot}51 \pm 1{\cdot}77$	$24{\cdot}42 \pm 2{\cdot}86$	$22{\cdot}33 \pm 1{\cdot}91$	$26{\cdot}47 \pm 3{\cdot}47$
M_T (g) (T,*,§)	$84{\cdot}83 \pm 24{\cdot}57$	$160{\cdot}27 \pm 59{\cdot}86$	$105{\cdot}60 \pm 29{\cdot}19$	$207{\cdot}58 \pm 86{\cdot}25$
I_G (S,T,*,§)	$0{\cdot}04 \pm 0{\cdot}13$	$0{\cdot}17 \pm 0{\cdot}05$	$-0{\cdot}11 \pm 0{\cdot}17$	$-0{\cdot}08 \pm 0{\cdot}21$
I_H (T,*,§)	$0{\cdot}03 \pm 0{\cdot}12$	$-0{\cdot}06 \pm 0{\cdot}13$	$0{\cdot}07 \pm 0{\cdot}12$	$-6{\cdot}10^{-3} \pm 0{\cdot}09$
I_S (S,T,*,§)	$0{\cdot}05 \pm 0{\cdot}18$	$0{\cdot}02 \pm 0{\cdot}06$	$-0{\cdot}03 \pm 0{\cdot}10$	$-0{\cdot}03 \pm 0{\cdot}10$
Lysozyme activity (T,*,§)	$20{\cdot}08 \pm 5{\cdot}91$	$11{\cdot}95 \pm 3{\cdot}39$	$23{\cdot}31 \pm 6{\cdot}87$	$12{\cdot}15 \pm 6{\cdot}19$
Oxidative burst efficiency (*,§)	$0{\cdot}50 \pm 0{\cdot}68$	$0{\cdot}65 \pm 0{\cdot}71$	$1{\cdot}12 \pm 0{\cdot}93$	$0{\cdot}44 \pm 0{\cdot}34$
Neutrophiles (*,§)	6·16	9·71	12·50	4·15
Blast cells (S)	1·14	1·14	0·16	0·23
Lymphocytes (*,§)	90·80	87·42	85·60	94·15
Monocytes (*,§)	1·33	1·71	1·66	1·46

L_T, total length; M_T, total mass; I_G, investment in gonads; I_H, investment in hepatopancreas; I_S, investment in spleen; S, significant effect ($P < 0{\cdot}05$) of sex; T, significant effect ($P < 0{\cdot}05$) of temperature; *, difference in temperatures within males; §, difference in temperature within females.

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TABLE II. Intensity of infection by different type of parasites for male (M) and female (F) *Leuciscus cephalus* for different temperatures. Values are minimum; median; maximum (in term of total number of parasites per host). Due to low intensity of infection by endoparasites in most of the individuals, only the total number of endoparasites is given (and was used in further analysis)

Sex	Males		Females	
Temperature (° C)	6·1	10·1	6·1	10·1
Gyrodactylus spp. (T,S,§)	0;9;22	2;4·5;25	4;10;20	3;20;10
Dactylogyrus spp. (T,S,*,§)	15;26;93	18;42;62	6;24·5;66	9;48;163
Ectoparasites (T,S,*,§)	17:39;101	23;48;75	12;37·5;92	16;51;170
Endoparasites (T,S,§)	0;1;7	0;1·5;13	0;0·5;6	0;4;13
Total (T,S,*,§)	18;42;102	24;52;88	12;40·5;92	16;63;170

Total, both endoparasites and ectoparasites; S, significant effect ($P < 0{\cdot}05$) of sex; T, significant effect ($P < 0{\cdot}05$) of temperature; *, difference between temperatures within males; §, difference between temperatures within females.

the variability in the data set when PCA was performed using the first temperature sample (6·1° C). Using data from the second temperature sample (10·1° C), the first axis explained 37·71% and the second explained 19·43% of the variability in the data set. T Mann–Whitney *U*-test revealed no significant differences between males and females for measured variables (except for the indicator of investment in gonad mass, for which the two sexes were different, $P < 0{\cdot}001$).

At 6·1° C, the neutrophil count and respiratory burst intensity were positively associated, and both negatively correlated to parasitism and investment in gonad mass based on the results of first PCA [Fig 1(a)]. At 10·1° C, lysozyme activity and lymphocytes count were positively associated, and negatively associated with hepatopancreas, spleen and gonads investment, and neutrophil count (all of these variables were positively associated) based on the results of second PCA [Fig. (1b)]. Parasitism and respiratory burst intensity were positively associated, but independent of all other indicators. The *P*-values of correlations between all the variables used in Fig. 1 are given in Appendix II.

Temperature increased between the two sampling days from 6·1° C on 3 April to 10·1° C on 14 April. A significant two-fold decrease in mucus' lysozyme activity associated with higher temperature in the whole population was observed (Mann–Whitney *U*-test, $P < 0{\cdot}001$), in males ($P < 0{\cdot}05$) and in females ($P < 0{\cdot}05$) (Fig. 2). At 10·1° C, investment in gonad mass and lysozyme activity were negatively correlated ($r = -0{\cdot}4$, $F_{12,12}$, $P < 0{\cdot}05$). Lysozyme activity was not correlated with intensity of infection by monogeneans ($r = -0{\cdot}13$, $P > 0{\cdot}05$) or the composite value for parasitism ($r = -0{\cdot}2$, $P > 0{\cdot}05$).

Respiratory burst intensity was significantly higher in females than in males at 6·1° C (Mann–Whitney *U*-test, $P < 0{\cdot}05$). This intensity decreased in females and increased in males from 6·1 to 10·1° C, even if these fluctuations were not significant (both $P > 0{\cdot}05$).

At 10·1° C, there was a significant positive correlation between respiratory burst intensity and composite value for parasitism ($r = 0{\cdot}55$, $F_{20,20}$, $P < 0{\cdot}05$). Parasite load increased from 6·1 to 10·1° C, but this increase was non-significant

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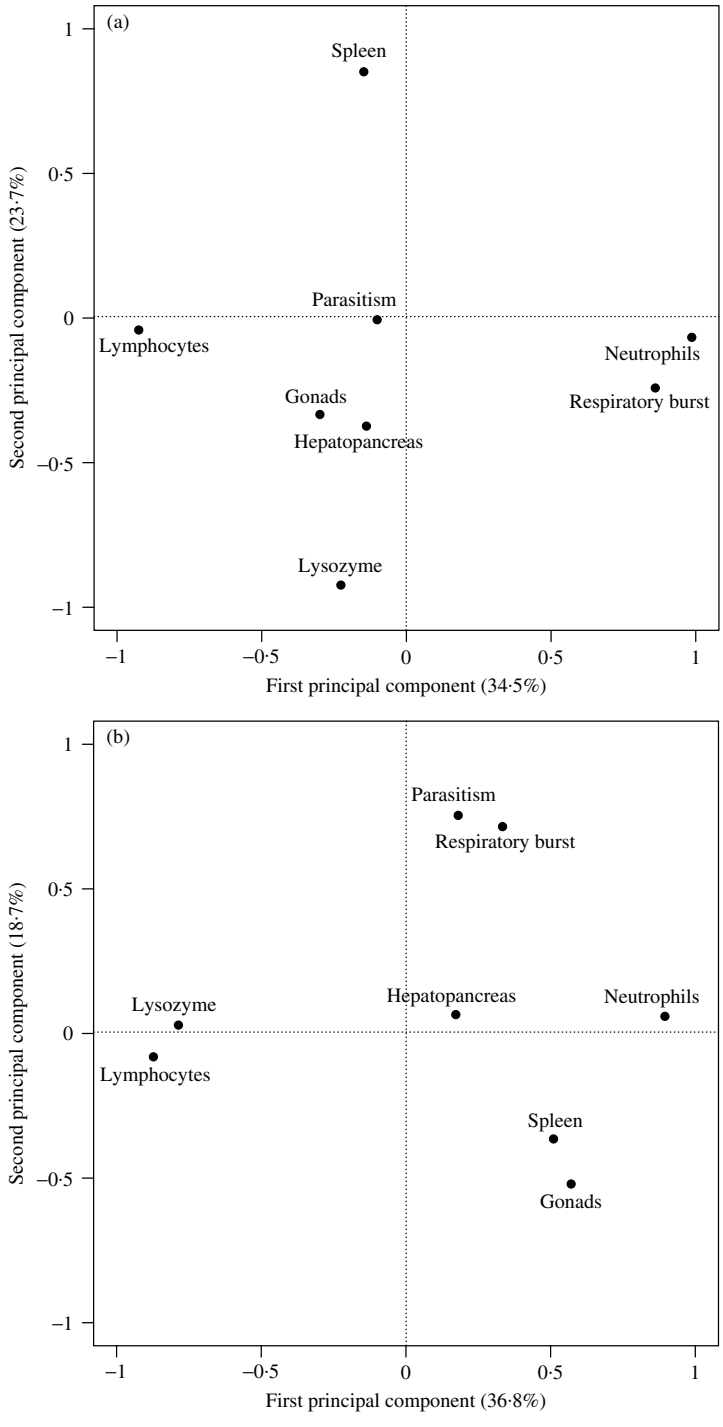

FIG. 1. Principal component analysis with the following *Leuciscus cephalus* variables: parasitism, lysozyme activity, respiratory burst efficiency, number of neutrophiles, number of lymphocytes, and investment in gonads, hepatopancreas and spleen mass at (a) 6·1 and (b) 10·1° C.

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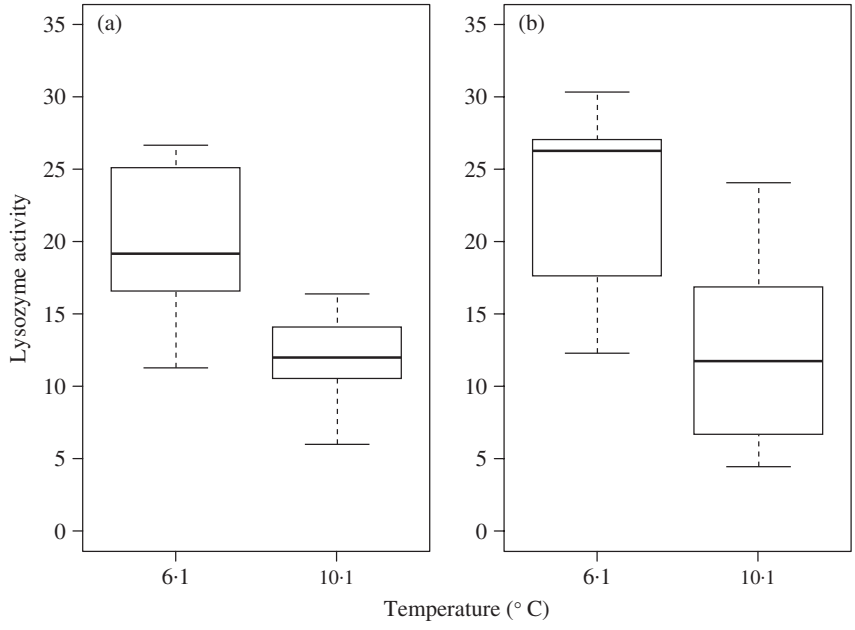

FIG. 2. Activity of lysozyme according to temperature, for (a) male and (b) female *Leuciscus cephalus*. ——, median. Boxes are the second and third quartiles, whiskers limits are lower value of first quartile, and upper value of fourth quartile.

(Mann–Whitney U-test, $P > 0{\cdot}05$) in the whole population. This increase was significant only in females ($P < 0{\cdot}05$) but not in males ($P > 0{\cdot}05$) (Fig. 3).

At 6·1° C, there was a significant positive correlation between neutrophil counts and oxidative burst intensity in the whole population ($r = 0{\cdot}84$ $F_{12,10}$, $P < 0{\cdot}001$). This correlation was recorded in females ($r = 0{\cdot}86$, $P < 0{\cdot}001$) but not in males ($P > 0{\cdot}05$). At 10·1° C, the neutrophil count and respiratory burst intensity were independent when analysing the whole population as well as in separated analyses for males and females. The neutrophil counts and respiratory burst intensity decreased from 6·1 to 10·1° C, but this decrease was significant only for neutrophil counts ($P < 0{\cdot}05$).

Interactions between indicators of immunocompetence were assessed using PCA (Fig. 4). At 6·1° C, the two principal components accounted for 75·6% of the variance among the indicators. At 10·1° C, the two principal components accounted for 63·5% of this variance. There was no pattern of correlation between the selected indicators.

DISCUSSION

As each organism must allocate a finite amount of energy between antagonistic activities, it is expected that trade-offs will occur between immunocompetence and other important life functions, mainly reproduction and somatic condition (Sheldon & Verhulst, 1996). Seminal studies have emphasized the strong implications

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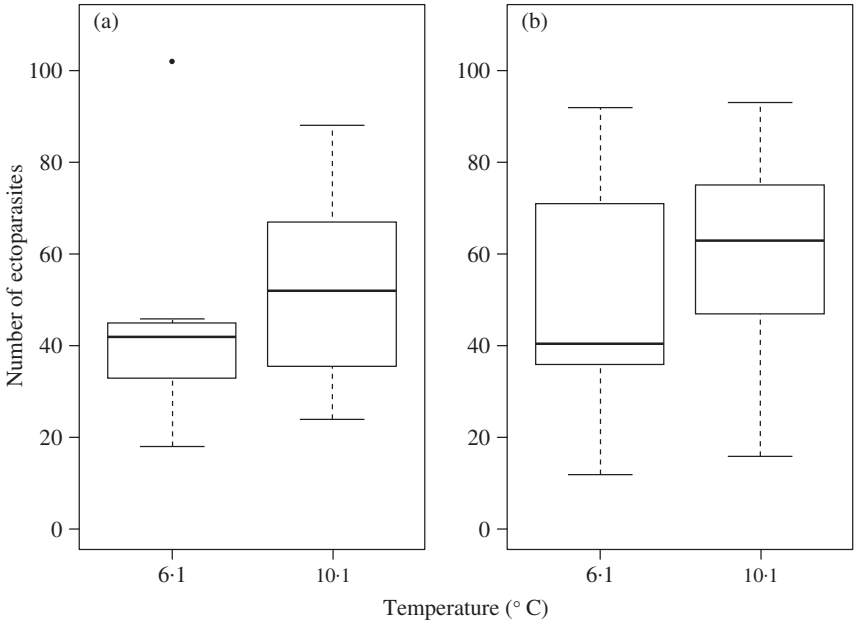

FIG. 3. Modification of the number of ectoparasites in (a) male and (b) female *Leuciscus cephalus* between 6·1 and 10·1° C. ——, median. Boxes are the second and third quartiles, whiskers limits are lower value of first quartile and upper value of fourth quartile. ●, outliers.

of trade-off analyses in evolutionary biology (Zahavi, 1975; Hamilton & Zuk, 1982; Folstad & Karter, 1992). These trade-offs, however, should not be considered as obvious, and more investigations are needed to provide evidence for their existence (Lochmiller & Deerenberg, 2000). Analyses of relationships between immune status and somatic condition will allow investigation of potential trade-offs in this model, especially those occurring between investments in immunocompetence and reproduction. Some of these trade-offs are likely to originate from interaction of biological functions. For example, the immunosuppressive role of testosterone in salmonids is well documented (Slater & Schreck, 1997), and it is known that blood composition fluctuates as a reflection of reproductive status in several fishes (Pickering, 1986). Vainikka *et al*. (2004, 2005) showed, however, that in the roach *Rutilus rutilus* (L.), testosterone level was positively correlated to immune defence efficiency, indicating that the putative trade-off between investment in reproduction and immunocompetence might not be strong in all fish species. There is still a need, however, to assess it in one of the most challenging periods of the year for the fishes. Moreover, as the putative immunosuppressive role of some aspects of reproduction is the basis of the hypothesis proposed by Folstad & Karter (1992), the study of such interactions is relevant to evolutionary biologists.

Sex-related traits influence fish immunocompetence and somatic condition (Vainikka *et al*., 2004). Investment in reproduction is different between males and females. Females invest in gonad mass, whereas males allocate some of their energy to sexual ornamentation (Schoen & Stewart, 1986; Folstad & Karter, 1992; Skarstein *et al*., 2001). Such differences in physiological functions and immune response

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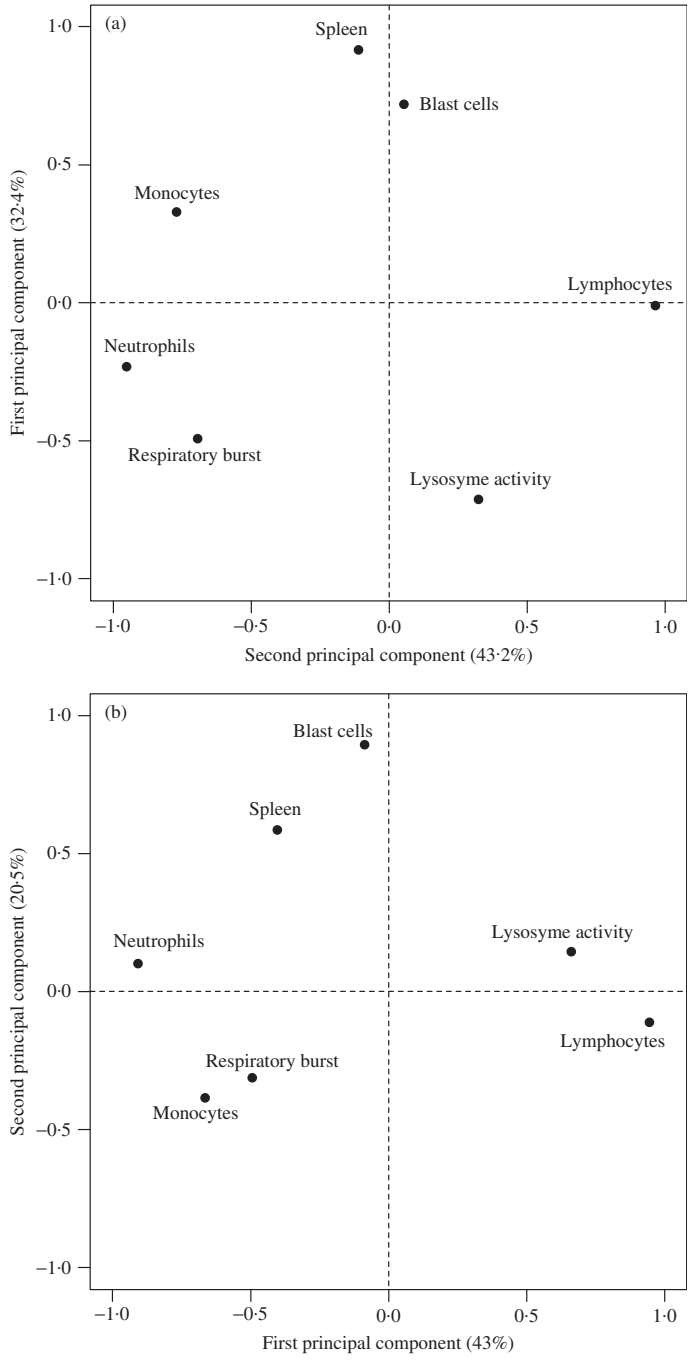

FIG. 4. Principal component analysis conducted on the variables involved in *Leuciscus cephalus* immunocompetence, and the index of investment in spleen mass at (a) 6·1 and (b) 10·1° C. The fact that variables are equally distributed indicates that their contributions to the overall immunocompetence are roughly equal, and that each one of them is important to consider in order to properly assess immunocompetence. Moreover, it appears that the spleen displays no correlation with other indicators.

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between males and females could also be related to seasonal variation of water temperature (Bly & Clem, 1991; Le Morvan *et al*., 1998). Gonado-somatic and spleno-somatic indices in *L. cephalus* were similar in males and females in spring and autumn (Lamkovà *et al*., 2007). Moreover, sex-related differences in immunocompetence and condition could be seen as a reflection of differences in endocrine activity, as those traits are involved in sexual selection, and hence strongly linked to hormonal status (Wingfield & Moore, 1987; Milinski & Bakker, 1990; Hillgarth & Wingfield, 1997). Until now, however, few data comparing the level of parasitism and immunocompetence and health status have been available for the winter period in cyprinids.

Elevation of temperature, occurring for example in early spring, is known to affect both life cycle and population dynamics of several fish parasites, such as gyrodactylid monogeneans (Gelnar, 1987; Jansen & Bakke, 1991; Andersen & Buchmann, 1998), as well as fish immune responses (Bly & Clem, 1991). The natural increase of temperature allows the study of interactions and variations of selected indices of immunocompetence, somatic condition and parasitism, during the winter to spring transition in a wild population of *L. cephalus*. The period in which this study occurred was therefore highly challenging: parasitic load showed a significant increase, investment in reproductive effort began and the environment was changing rapidly.

The elevation of temperature decreased lysozyme activity (Fig. 2) in contradiction with other studies (Bowden *et al*., 2004; Kumari *et al*., 2006), where lysozyme activity and temperature were positively correlated. It is generally accepted that higher temperatures lead to increased immunocompetence in poikilotherms, whereas lower temperatures are thought to lower immunocompetence (Raffel *et al*., 2006, Pérez-Casanova *et al*., 2008). While temperature increased, however, investment in gonad mass (as measured by residuals of expected gonad mass at a given body mass) increased concurrently, even if this increase was only significant for male fish ($P < 0{\cdot}05$). Lysozyme activity and investment in gonad mass were negatively correlated at $10{\cdot}1^{\circ}$ C, an indication of a potential trade-off in energy allocation between innate immunity and gonad mass in early spring. Increase in gonad mass between winter and spring as well as seasonal variations of several somatic condition indices have already been documented in *L. cephalus* (Lamkovà *et al*., 2007). Moreover, previously cited studies (Bowden *et al*., 2004; Kumari *et al*., 2006) took place over a larger time, at which short scale variations in selected indices, provoked by a punctual stress, could not be observed.

No significant correlation between lysozyme activity and parasitism was found. This may indicate that parasite load is not important enough (lower recruitment and death of parasites overwinter) in early spring and does not trigger an immune response, or that fish have only enough energy reserves to invest in gonads, and are therefore unable to mount an immune response against their parasites. The fact that parasite increase is only weakly significant seems to support this hypothesis. No significant correlation was found between intensity of infection by monogeneans and lysozyme activity, despite several reports of such a correlation (Ingram, 1980; Buchmann, 1999; Buchmann & Lindenstrøm, 2002). Again, this lack of correlation could be the result of the effects of both temperature and increase in gonad investment.

There was a significant positive correlation between respiratory burst intensity and parasitism, but there was difference between the sexes. Parasitic load increased from

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6·1 to 10·1° C, especially for ectoparasites (Fig. 3), but this was only significant in females. The fact that their generation time is shorter than endoparasites, and that ectoparasites are more sensitive to temperature could explain this observation. The respiratory burst intensity decreased in females and simultaneously increased in males, although these changes were not significant. The weak effect of temperature on respiratory burst was already suggested by Pérez-Casanova *et al*. (2008). At 10·1° C, a weakly significant positive correlation between respiratory burst intensity and parasitism was found both in males and females. It could be hypothesized that an increase in water temperature affects both parasites and hosts, leading to enhancement of both parasite development and host investment in immunity (here mediated by the respiratory burst capacity of phagocytes). No correlation was found between respiratory burst intensity and investment in gonads, suggesting that all immune mechanisms were not equally dependent on energy resources, and that some of them may be maintained even when available energy is scarce (as at low temperatures).

A significant positive correlation was found between respiratory burst intensity and neutrophil counts in the whole population at 6·1° C, but not at 10·1° C. Neutrophils (as well as monocytes) are responsible for respiratory burst. This suggests that at low temperature, neutrophils are mobilized to participate in respiratory bursts, giving an efficient response against parasites. When water temperature increases, *i.e*. when environmental stress is reduced, however, neutrophils could be involved in other functions in addition to anti-parasite defence. The observation that those two measurements of immunity decreased during temperature elevation may indicate that higher temperature leads fish to divert an important part of their energy to gonad mass (*i.e*. in reproductive function) in early spring. A comparison of neutrophil activity under different temperature conditions, however, is required to confirm this supposition.

The PCA results indicated that indices of gonad and spleen investment were correlated, especially at higher temperature. No significant correlations were found between investment in spleen and other measurements of immune system activity (Fig. 4) or parasitism. This result was also found in other cyprinid species by Vainikka *et al*. (2009). It is not possible, however, to rule out the possibility that the lack of correlation was specific to this period of the year, or that spleen response was driven by microparasites, which were not investigated.

No clear interactions or correlations between immunocompetence indices selected in this study were shown (Fig. 4). This indicates that relying on a single measurement to assess immunocompetence, as is frequently done, is insufficient, and that mechanisms underlying immune response against parasites need further investigation for selecting relevant indices. For example, doing a global assessment of interactions between parasitism, life traits and immune defence in 24 species of freshwater cyprinids, Simkova *et al*. (2008) found no negative correlation between spleen size and gonad size in males, which seemed contradictory with the immunohandicap hypothesis. This contradiction is only true if it is assumed that spleen size (or the spleno-somatic index) is a relevant index of immunocompetence in fishes (National Research Council, 1992), a hypothesis which is challenged by the results presented during this work. Moreover, it may be important to focus on the potential differences at the intraspecific or interspecific ranges in further studies.

The present study suggested that early spring investment in reproductive function (measured by increase in gonad mass) was detrimental to immunity (activity

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of lysozyme and respiratory burst intensity). Moreover, immunocompetence was strongly dependent upon the environment, the key factor being water temperature. Trade-offs between immunocompetence and other physiological functions (*e.g.* investment in reproduction or somatic maintenance) are likely to be regulated by both parasitism and environmental factors.

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References

Andersen, P. S. & Buchmann, K. (1998). Temperature dependent population growth of *Gyrodactylus derjavini* on rainbow trout, *Oncorhynchus mykiss*. *Journal of Helminthology* **72,** 9–14.

Blazer, V. (1991). Piscine macrophage function and nutritional influences: a review. *Journal of Aquatic Animal Health* **32,** 77–86.

Bly, J. & Clem, L. (1991). Temperature-mediated processes in teleost immunity: *in vitro* immunosuppression induced by *in vivo* low temperature in channel catfish. *Veterinary Immunology and Immunopathology* **28,** 365–377.

Bolger, T. & Connolly, P. L. (1989). The selection of suitable indices for the measurement and analysis of fish condition. *Journal of Fish Biology* **34,** 171–182.

Bowden, T. J., Butler, R. & Bricknell, I. R. (2004). Seasonal variation of serum lysozyme levels in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish & Shellfish Immunology* **17,** 129–135.

Buchmann, K. (1998). Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). *Diseases of Aquatic Organisms* **32,** 195–200.

Buchmann, K. (1999). Immune mechanisms in fish skin against monogeneans – a model. *Folia Parasitologica (Praha)* **46,** 1–9.

Buchmann, K. & Bresciani, J. (1998). Microenvironment of *Gyrodactylus derjavini* on rainbow trout Oncorhynchus mykiss: association between mucous cell density in skin and site selection. *Parasitology Research* **84,** 17–24.

Buchmann, K. & Lindenstrøm, T. (2002). Interactions between monogenean parasites and their fish hosts. *International Journal for Parasitology* **32,** 309–319.

Buchmann, K. & Uldal, A. (1997). *Gyrodactylus derjavini* infections in four salmonids: Comparative host susceptibility and site selection of parasites. *Diseases of Aquatic Organisms* **28,** 201–209.

Crofton, H. D. (1971). A quantitaitve approach to parasitism. *Parasitology* **62,** 179–193.

Ergens, R. & Lom, J. (1970). *Causative Agents of Parasitic Fish Diseases*. Praha: Academia Praha.

Folstad, I. & Karter, A. J. (1992). Parasites, bright males, and the immunocompetence handicap. *The American Naturalist* **139,** 603–622.

Gelnar, M. (1987). Experimental verification of the effect of water temperature on micropopulation growth of *Gyrodactylus katharineri* Malmberg, 1964 (Monogenea) paraziting carp fry (*Cyprinus carpio* L.). *Folia Parasitologica* **34,** 19–23.

Georgiev, B., Biserkov, V. & Genov, T. (1986). *In toto* staining method for cestodes with iron acetocarmine. *Helminthologia* **23,** 279–281.

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Gusev, A. (1985). Metazoan parasites Part I. In *Identification Key to Parasites of Freshwater Fish* (Bauer, O., ed.), pp. 1–424. Leningrad: Nauka (in Russian).
Hamilton, W. & Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? *Science* **218,** 384–387.
Harris, P., Soleng, A. & Bakke, T. (1998). Killing of *Gyrodactylus salaris* (Platyhelminthes, Monogenea) mediated by host complement. *Parasitology* **117,** 137–143.
Hillgarth, N. & Wingfield, J. (1997). Parasite-mediated sexual selection: endocrine aspects. In *Host-parasite Evolution: General Principles and Avian Models* (Clayton, D. H. & Moor, J., eds), pp. 78–104. Oxford: Oxford University Press.
Ingram, G. A. (1980). Substances involved in the natural resistance of fish to infection – a review. *Journal of Fish Biology* **16,** 23–60.
Jansen, P. A. & Bakke, T. A. (1991). Temperature-dependent reproduction and survival of *Gyrodactylus salaris* Malmberg, 1957 (Platyhelminthes: Monogenea) on Atlantic salmon (*Salmo salar* L.). *Parasitology* **102,** 105–112.
Jones, S. R. (2001). The occurrence and mechanisms of innate immunity against parasites in fish. *Developmental Comparative Immunology* **25,** 841–852.
Kuby, J. (1999). Cells and organs of the immune system. In *Immunology*, 5th edn (Goldsby, R. A., Kindt, T. K., Osborne, B. A. & Kuby, J., eds), p. 44. New York, NY: W.H. Freeman & Co.
Kumari, J., Sahoo, P. K., Swain, T., Sahoo, S. K., Sahu, A. K. & Mohanty, B. R. (2006). Seasonal variation in the innate immune parameters of the Asian catfish *Clarias batrachus*. *Aquaculture* **252,** 121–127.
Kurtz, J. (2007). Evolutionary ecology of immune defence in copepods. *Plankton Research* (Suppl. 1) **29,** i27–i38.
Lamkovà, K., Šimkovà, A., Palikova, M., Jurajda, P. & Lojek, A. (2007). Seasonal changes of immunocompetence and parasitism in chub (*Leuciscus cephalus*), a freshwater cyprinid fish. *Parasitology Research* **101,** 775–789.
Le Morvan, C., Troutaud, D. & Deschaux, P. (1998). Differential effects of temperature on specific and nonspecific immune defences in fish. *Journal of Experimental Biology* **201,** 165–168.
Lehmann, J., Hamers, R. & Stürenberg, F.-J. (1994). *Hämatologie des Karpfens* – Cyprinus carpio. Berlin: Landesanstalt für Fischerei NRW, Fischphysiologie.
Lochmiller, R. L. & Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88,** 87–98.
Lusková, V. (1997). Annual cycles and normal values of hematological parameters in fishes. *Acta Scientiarum Naturalium Academiae Scientiarum Bohemicae Brno* **31,** 1–70.
Milinski, M. & Bakker, T. (1990). Female stickelbacks use male coloration in mate choice and hence avoid parasitized males. *Nature* **344,** 330–333.
Møller, A., Christe, P., Erritzoe, J. & Mavarez, J. (1998). Condition, diseases and immune defence. *Oikos* **83,** 301–306.
Moravec, F. (1994). *Parasitic Nematodes of Freshwater Fishes of Europe*. Praha: Academia Praha.
Moravec, F. (2001). *Checklist of Metazoan Parasites of Fishes of the Czech Republic and the Slovak Republic (1873-2000)*. Praha: Academia Praha.
Mustafa, A., MacWilliams, C., Fernandez, N., Matchett, K., Conboy, G. A. & Burka, J. F. (2000). Effects of sea lice (*Lepeophtheirus salmonis* Kröyer, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar* L.). *Fish and Shellfish Immunology* **10,** 47–59.
National Research Council (1992). *Biologic Markers in Immunotoxicology*. Washington, DC: National Academy Press.
Nikoskelainen, S., Nuutila, J. & Lilius, E.-M. (2004). *Luminescence and Flow-Cytometry Applications for Fish Immunology*. Turku, Finland: University of Turku.
Pérez-Casanova, J. C., Rise, M. L., Dixonb, B., Afonso, L. O. B., Hall, J. R., Johnson, S. C. & Gamperl, A. K. (2008). The immune and stress responses of Atlantic cod to long-term increases in water temperature. *Fish and Shellfish Immunology* **24,** 600–609.
Pickering, A. D. (1986). Changes in blood cell composition of the brown trout, *Salmo trutta* L., during the spawning season. *Journal of Fish Biology* **29,** 335–347.

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Raffel, T. R., Rohr J. R., Kiesecker J. M. & Hudson P. J. (2006). Negative effects of changing temperature on amphibian immunity under field conditions. *Functional Ecology* **20,** 819–828.

Rubio-Godoy, M., Porter, R. & Tinsley, R. (2004). Evidence of complement-mediated killing of *Discocotyle sagittata* (Platyhelminthes, Monogenea) oncomiracidia. *Fish and Shellfish Immunology* **17,** 95–103.

Schoen, D. & Stewart, S. (1986). Variation in male reproductive investment and male reproductive success in white spruce. *Evolution* **40,** 1109–1120.

Scholz, T. (1989). Amphilinida and Cestoda, parasites of fish in Czechoslovakia. *Acta Scientiarum Naturalium Academiae Scientiarum Bohemicae Brno* **23,** 1–56.

Sheldon, B. & Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution* **11,** 317–321.

Simkova, A., Lafond, T., Ondrackova, M., Jurajda, P., Ottova, E. & Morand, S. (2008). Parasitism, life history traits and immune defence in cyprinid fish from Central Europe. *BMC Evolutionary Biology* **8,** 29.

Skarstein, F., Folstad, I. & Liljedal, S. (2001). Whether to reproduce or not: immune suppression and costs of parasites during reproduction in the Arctic charr. *Canadian Journal of Zoology* **79,** 271–278.

Slater, C. & Schreck, C. (1997). Physiological levels of testosterone kill salmonid leukocytes *in vitro*. *General and Comparative Endocrinology* **106,** 113–119.

Stearns, S. (1976). Life-history tactics: a review of the ideas. *The Quarterly Review of Biology* **51,** 3–4.

Stolen, J., Fletcher, T., Anderson, P., Kaattari, S. & Rowley, A. (1993). *Techniques in Fish Immunology*. Fair Haven, NJ: SOS Publications.

Svobodová, Z., Pravda, D. & Paláčková, J. (Eds). (1986). *Universal Methods of Hematological Investigations in Fish*. VÚRH Vodňany, Czech Republic: Edition Metodik.

Svobodová, Z., Pravda, D. & Paláčková, J. (1991). Unified methods of haematological examination of fish. *Manuals of Research Institute of Fish Culture and Hydrobiology 22*. Vodňany, Czech Republic: University of South Bohemia.

Vainikka, A., Jokinen, E. I., Kortet, R. & Taskinen, J. (2004). Gender- and season-dependent relationships between testosterone, oestradiol and immune functions in wild roach. *Journal of Fish Biology* **64,** 227–240.

Vainikka, A., Jokinen, E. I., Kortet, R., Paukku, S., Pirhonen, J., Rantala, M. J. & Taskinen, J. (2005). Effects of testosterone and beta-glucan on immune functions in tench. *Journal of Fish Biology* **66,** 348–361.

Vainikka, A., Taskinen, J., Lötynoja, K., Jokinen, E. I. & Kortet, R. (2009). Measured immunocompetence relates to the proportion of dead parasites in a wild roach population. *Functional Ecology* **23,** 187–195.

Watts, M., Munday, B. L. & Burke, C. M. (2001). Immune responses of teleost fish. *Australian Veterinary Journal* **79,** 570–574.

Wingfield, J. & Moore, M. (1987). Hormonal, social, and environmental factors in the reproductive biology of free-living male birds. In *Psychobiology of Reproductive Behavior: An Evolutionary Perspective* (Wingfield, J. & Moore, M., eds), pp. 149–175. Englewood Cliff, NJ: Prentice Hall.

Zahavi, A. (1975). Mate selection – a selection for a handicap. *Journal of Theoretical Biology* **53,** 205–214.

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APPENDIX I. List of parasite species found during the investigation

Dactylogyrus	*Gyrodactylus*	Other species
D. fallax	*G. decorus*	*Myxobolus* sp.
D. folkmanovae	*G. osoblahensis*	*Anodonta* sp.
D. micracanthus	*G. leucisci*	*Proteocephalus torulosus*
D. nanoides	*G. vimbi*	*Neoechinorynchus rutili*
D. nanus	*G. prostae*	*Pomphorynchus laevis*
D. naviculoides	*G. lomi*	*Ergasilus sieboldi*
D. prostae	*G. lamberti*	
D. similes	*G. kearni*	
D. sphyrna	*G. gracilihamatus*	
D. vistulae	*G. gasterostei*	
D. vranoviensis	*G. scardinensis*	

APPENDIX II. *P*-values of Pearson's correlation test of variables used in Fig. 1 at 6·1 and 10·1° C (respectively above and below the diagonal). Positive correlations are given in bold. Non-significant *P*-values ($P > 0{\cdot}05$) are not given

	Spleen	Gonads	Hepatopancreas	Lymphocytes	Neutrophils	Lysozyme	Respiratory burst	Parasitism
Spleen	—							
Gonads	<**0·001**	—	<**0·05**					
Hepatopancreas	<**0·05**	<**0·001**	—					
Lymphocytes				—	<0·001		0·01	
Neutrophils				< 0·001	—		<**0·001**	
Lysozyme				<**0·05**	0·01	—		
Respiratory burst				0·01	<**0·05**		—	
Parasitism								—

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