Measured immunocompetence relates to the proportion of dead parasites in a wild roach population

Anssi Vainikka, Jouni Taskinen, Katja Löytynoja, E. Ilmari Jokinen and Raine Kortet

Summary

1. Although various methods are used to measure immunocompetence, their relationship with the actual parasite clearance or parasite load is seldom demonstrated in natural systems.

2. We combined nine measures of immune function using principal component analysis (PCA), and examined the relationship of the collective measures with (i) the proportion of parasites killed by the host, (ii) the burden of several parasite species and (iii) a viral disease in a wild population of the roach, Rutilus rutilus. We also studied if these variables were associated with the concentration of steroids (testosterone and oestradiol).

3. Most significant correlations between the loads of ecto- and gill parasites and the measures of immunity were positive, suggesting either a parasite-induced immunoactivation or temporal covariation. When the temporal covariation was statistically removed, the functional aspects (respiratory burst, chemotaxis) of phagocytotic cells and the total concentration of immunoglobulin M correlated positively with the proportion of dead Rhipidocotyle campanula (Digenea) parasites.

4. However, no relationships remained between the parasite loads and the measures of immunity or concentrations of the hormones.

5. The present results suggest that the measures of phagocytotic activity and IgM concentrations relate to an observable competence of roach to eliminate parasites. However, new experimental approaches are needed to reveal the causal mechanisms behind the sometimes ambiguous correlations.

Key-words: immune defence, innate immunity, parasite resistance, trade-off

Introduction

Parasites and pathogens impose strong natural selection pressures on their hosts (Clarke 1979; Price 1980; Goater & Holmes 1997), and therefore, efficient immune function may be favoured both by natural and sexual selection (Hamilton & Zuk 1982; Andersson 1994). Handicapping sexual signalling (Zahavi 1975, 1977) and unavoidable energetic trade-offs between immune function and reproduction link parasite load to the fitness of a host, mainly via endocrinological mechanisms (Folstad & Karter 1992; Wedekind & Folstad 1994; Kurz et al. 2007). In immunological ecology, various methods are used to estimate individuals’ immunocompetence (e.g. Skarstein, Folstad & Liljedahl 2001; Kortet et al. 2003a; Måsvær, Liljedahl & Folstad 2004). However, in addition to the rather well-established connection between specific antibodies and disease resistance in vertebrates (Casadevall 2004), surprisingly few data show how the commonly used non-specific measures of immune function actually relate to abundance of parasites and diseases, or to individuals’ ability to control the cost of parasitism (e.g. Pulsford et al. 1995; Sakai et al. 1996; Hakoyama et al. 2001).

It is clear that more detailed knowledge on the relationships between the measured indices of immune function and parasite load and parasite resistance in natural systems is needed to explain the causal mechanisms of these interactions, and to reveal whether high measures of immunity are protective against parasitic infections. Studies of this kind could also help us to estimate the ecological consequences of stress and pollutants that affect several immune functions (Pulsford et al. 1995). Moreover, specific information on how different aspects of immune function are related to parasite load in the wild is a necessary background for future interpretation of optimal immune responses (Viney, Riley & Buchanan 2005).

Within an individual’s life-history, the development, maintenance and activation of immune defence can be considered as a significant sink of energy that is traded against growth and reproduction (e.g. Tschirren & Richner 2006; Simková et al. 2008). Since many life-history traits are physiologically regulated by hormonal mechanisms, these trade-offs are often detected as correlations between concentrations of
reproductive hormones and measures of immune function or parasite loads (Hillgarth & Wingfield 1997; Braude, Tang-Martinez & Taylor 1999; Kortet & Vainikka 2008). However, the sign of correlations between sex hormone concentrations, measures of non-specific immunity and parasite loads is often difficult to predict, since many relationships are bi-directional and may be shaped by the efficiency of the adaptive immunity determined by the set of available MHC-alleles (Schmid-Hempel & Ebert 2003; Kurtz et al. 2004; Ottová, Šimková & Morand 2007). Therefore, additional descriptive information about patterns in parasite–immune defence interactions is needed in wild animals.

Males of many cyprinids signal both parasite resistance (Taskinen & Kortet 2002; Kortet et al. 2004) and parasite load (Wedekind 1992; Kortet & Taskinen 2004; Ottová et al. 2005) to females by the expression of androgen-dependent secondary sexual characters; breeding tubercles (Kortet et al. 2003b). Previously, we found using the same data as in the current study, that individual measures of immunity of roach, Rutillus rutillus, showed seasonal changes that to some extent related to reproduction (Kortet et al. 2003a). However, the concentrations of testosterone and oestradiol were not negatively, but often positively, correlated with the individual measures of immune function (Vainikka et al. 2004a).

Here, we present novel data on parasite abundances on roach sampled for the study of Kortet et al. (2003a) and further analysed for the correlations between the measures of immunity and sex hormone concentrations in Vainikka et al. (2004a). We reanalyse the old data by combining the nine measures of immune function (relative counts of (1) lymphocytes, (2) thrombocytes, (3) granulocytes and (4) macrophages, (5) total IgM concentration, (6) relative mass of the spleen, (7) total count of leukocytes in blood, (8) chemiluminescence and (9) migration differential of head kidney phagocytes) into principal components and comparing their expression between sexes during the year. The main aim of this study is to examine the relationships between the combined measures of immunity and (1) load of several parasite species within and among seasons, (2) proportion of dead Raphidascaris acus and Rhipidocotyle campanula parasites, (3) occurrence of a viral disease, and finally (4) testosterone and oestriadiol concentrations. We aim to describe the relationships between the measures of immunity and the occurrence of parasites, and dead parasites, thereby potentially revealing which measures of immunity relate to the host’s ability to eliminate parasites.

Materials and methods

Roach (N = 149, length 158.8 ± 21.4 mm, mass 55.8 ± 27.2 g, mean ± SD) were captured by angling in five occasions (approximately 15 males and 15 females per time) during the year 1999 from Lake Jyväsjärvi, Jyväskylä, Finland. Fish were reared in accordance with the Guidelines for the Use of Animals in Research (Anon. 1996) under license from the Central Finland Regional Environmental Center (permission LS-299). Methods for fish sampling and physiological investigations are given in more detail in Kortet et al. (2003a) and Vainikka et al. (2004a).

Analyses of immunity and hormone levels

An overview of the methods is as follows; the fish were anesthetized immediately after capture using 0.01% MS-222 (Sigma Chemical Co., St Louis, MO), transported to the laboratory in aerated lake water and kept at the ambient temperature of the lake before examination not later than 2 h after capture. Whole blood (50 μL) was stained with Shaw dye for enumeration of red and white blood cells (Shaw 1930), and plasma was separated by centrifugation and frozen for the later determination of immunoglobulin M (IgM) and steroid concentrations. IgM was analysed using an enzyme-linked immunosorbent assay (ELISA) for roach IgM (Aaltonen, Jokinen & Valtonen 1994; Vainikka et al. 2004a). Spleen somatic index was calculated as the mass of the spleen the total body mass. Sex was identified from gonads. The chemiluminescence and migration of head kidney phagocytes (Scott & Klesius 1981) were assayed as described in Kortet et al. (2003a). The difference between spontaneous and directed migration (chemotactic differential) was used in the analyses. Plasma hormone concentrations were determined using RIA-based kits (TESTO-CTK, DiaSorin, Italy, for testosterone and ESTR-CTK-4, DiaSorin, Italy, for 17β-oestradiol).

Parasites and diseases

Mucous was collected from gills and skin of the fish using a scalpel, pressed on a slide under cover glass, and examined directly for Chilophora protozoan parasites with a microscope using transmitted light and 400× magnification. Parasite infestations from the gills, fins and liver were examined microscopically for Sporozoa protozoans and metazoan parasites by pressing the tissues between two large glass plates and using transmitted light with 100× magnification. We found Apisoma, Trichodina, Scyphidia and Ichthyophthirius multifiliis, chiliphores from the mucous of the gills and skin. Their numbers were estimated as a mean of three visual fields. I. multifiliis, known as the white spot disease, occurred so rarely that it was not included in further analyses. Instead, the sum of Apisoma-, Trichodina- and Scyphidia-type chiliphores, named as ‘ectoproteozoa on gills and epidermis’ (Table 1) was used in the statistical analyses. Pooling of these parasites is justified as they co-occur and have a similar way of living; they usually forage on bacterial flora and organic particles from the water but may start utilizing epidermal cells of fish and cause damage to skin when occurring in high numbers. These protozoans are moderately common parasites of roach in the present study area (Halmekoja, Valtonen & Taskinen 1992), and their numbers are known to respond to changes in host susceptibility, for example due to pollution (Lehtinen 1989; Khan et al. 1994). We encountered three sporozoan-type protozoan taxa; the myxozoans Myxobolus sp. from the gills, Myxobolus mulleri from the liver and an unidentified sporozoan from the fins. M. mulleri in the liver was very rare and was therefore excluded from further analyses. These sporozoans, named as ‘endoproteozoa’ (Table 1), are abundant among roach in the present study area (Brummer Koivunen, Tellervo Valtonen & Pugachev 1991) and can be harmful to the fish host (Shul’man 1990).

Among metazoan parasites, we found seven taxa belonging to the phylum Platyhelminthes within the gills, one of them being Dactylogyrus spp. (Monogenea);the sum of different Dactylogyrus species occurring on the gills of roach in the present study area (Table 1) (Koskivaara & Valtonen 1992). High abundance of Dactylogyrus spp. has also been suggested to be associated with pollution-induced impacts on the immune defence of roach (Valtonen, Holmes & Koskivaara 1997). Another monogenean in the gills, Paradiplozoon
Parasites and immune function in roach

Table 1. Prevalence, mean abundance and mean intensity for the three parasite species used in parametric analyses (three uppermost rows) and several mostly ectoparasitic, and less abundant species not included in parametric tests (lowermost rows). CI stands for the confidence interval.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Mean Prevalence</th>
<th>95% CI Prevalence</th>
<th>Mean Abundance</th>
<th>95% CI Abundance</th>
<th>Mean Intensity</th>
<th>95% CI Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhipidocotyle campamula</td>
<td>94.6</td>
<td>89.7–97.7</td>
<td>9.4</td>
<td>8.1–10.8</td>
<td>10.0</td>
<td>8.6–11.3</td>
</tr>
<tr>
<td>Rhipidocotyle fennica</td>
<td>88.4</td>
<td>82.1–93.1</td>
<td>28.5</td>
<td>21.8–35.2</td>
<td>32.3</td>
<td>24.9–39.6</td>
</tr>
<tr>
<td>Raphidascaris acus</td>
<td>84.6</td>
<td>77.7–90.0</td>
<td>14.4</td>
<td>10.2–18.5</td>
<td>17.0</td>
<td>12.2–21.8</td>
</tr>
<tr>
<td>Diplomastoma spathaceum</td>
<td>84.6</td>
<td>77.7–90.0</td>
<td>5.2</td>
<td>2.7–7.8</td>
<td>6.2</td>
<td>3.2–9.1</td>
</tr>
<tr>
<td>Ectoproteozoa on gills and epidermis</td>
<td>29.1</td>
<td>21.9–37.1</td>
<td>1.2</td>
<td>0.4–2.0</td>
<td>4.1</td>
<td>1.7–6.6</td>
</tr>
<tr>
<td>Monogenoa on gills</td>
<td>48.3</td>
<td>40.1–56.6</td>
<td>4.7</td>
<td>3.3–6.2</td>
<td>9.8</td>
<td>7.3–12.3</td>
</tr>
<tr>
<td>Ergasilus briani on gills</td>
<td>41.6</td>
<td>33.6–50.0</td>
<td>2.3</td>
<td>1.5–3.2</td>
<td>5.6</td>
<td>3.8–7.4</td>
</tr>
<tr>
<td>Myxobolus on gills</td>
<td>30.2</td>
<td>23.0–38.2</td>
<td>1.2</td>
<td>0.7–1.7</td>
<td>4.0</td>
<td>2.7–5.4</td>
</tr>
<tr>
<td>Tylodelphus clavata</td>
<td>23.5</td>
<td>16.9–31.1</td>
<td>1.4</td>
<td>0.4–2.4</td>
<td>6.1</td>
<td>2.1–10.1</td>
</tr>
<tr>
<td>Endosporoza</td>
<td>7.4</td>
<td>3.7–12.8</td>
<td>0.3</td>
<td>0.1–0.5</td>
<td>3.9</td>
<td>1.5–6.3</td>
</tr>
<tr>
<td>Neorhaphias japonicus</td>
<td>53.7</td>
<td>45.4–61.9</td>
<td>3.4</td>
<td>2.3–4.5</td>
<td>6.3</td>
<td>4.5–8.1</td>
</tr>
<tr>
<td>Glochidia total</td>
<td>18.8</td>
<td>12.9–26.0</td>
<td>0.5</td>
<td>0.3–0.7</td>
<td>2.6</td>
<td>1.7–3.6</td>
</tr>
</tbody>
</table>

homoion, was present too infrequently to be included in the analyses. Bucephalid digenean Rhipidocotyle campamula was encountered on the gills and R. fennica on the fins. Numbers of R. fennica were counted on the tail of the fish, as it appears to be the main site of infection for this species, whereas R. campamula infects almost exclusively the gills (Taskinen, Valtonen & Gibson 1991; Gibson, Valtonen & Taskinen 1992). These Rhipidocotyle species are the most common and abundant parasites of roach in the study area (Valtonen et al. 1997; Taskinen & Kortet 2002), and roach develops acquired immunity against Rhipidocotyle parasites (Aaltonen, Valtonen & Jokinen 1997). Bucephalid digeneans can be harmful to a fish host (Hoffmann et al. 1990); R. campamula can interrupt the blood circulation in the gills. Dead and live specimens of R. campamula in the gills of roach were identified as described by Taskinen & Kortet (2002). Among the observed digenean eye flukes, Diplomastoma spathaceum and Tylodelphus clavata, the former is known to impair vision of fish by causing cataracts (e.g., Karvonen, Seppälä & Valtonen 2004). For the nematode Raphidascaris acus, which damages liver tissue (Valtonen, Haaparanta & Hoffmann 1994), we observed both living (moving) and dead (encapsulated, motionless, degenerated) individuals. Among ectoparasites, the crustacean parasites Ergasilus sieboldi and Neorhaphias japonicus on skin occurred frequently, together with the unionid bivalve glochidia. These findings are in accordance with a previous study on roach in the same area (Tuuha, Valtonen & Taskinen 1992).

Three of the most prevalent species were used in the parametric analyses between the measures of immune function, hormone concentrations and parasite abundance (Table 1). Furthermore, nine parasite taxa (or groups) (prevalence 74–84.6%), and the proportion of dead R. acus individuals were used in non-parametric correlations between the measures of immune function, hormone concentrations and parasite abundance (Table 1). The proportion of dead R. campamula parasites of all R. campamula was not defined if the total number of parasites was less than three (thus N = 118). A similar criterion was used for dead R. acus; thus N = 82. The proportion of dead specimens was used as a proxy of the host’s ability to eliminate these parasites. However, the ratio is susceptible to uneven exposure to these parasites, and the delay after which dead specimens disappear is not known. These facts set further constrains for the interpretation.

Occurrence of the viral disease, epidermal papillomatosis, was examined visually and by hand (Korkea-Aho, Vainikka & Taskinen 2006a). Recent studies indicate that epidermal papillomatosis is associated with pollution and stress in roach (Korkea-Aho et al. 2006b, 2008).

STATISTICAL ANALYSES

Prior to analyses spleen-somatic index, relative proportions of differential leukocyte counts and the proportion of dead R. campamula and R. acus parasites were arcsine square root transformed. However, the proportion of dead R. acus was not successfully normalised and was therefore used only for non-parametric analyses. All other parameters and parasite counts were Ln(X + 1) transformed, but only the total load of R. campamula, R. fennica, and R. acus met the assumption of normality after transformation, and so only these were used in the parametric analyses. Principal component analysis was run on all nine immunological parameters, and principal components (for this on immunological components, IC: s) having eigenvalues over one (IC1, IC2, IC3 and IC4) were considered for further analysis. Correlations between hormones, measures of immune function and successfully normalised parasite intensities were first examined within each sex and period separately. The individual correlation coefficients were then meta-analysed as described in Hedges & Olkin (1985) to achieve 95% confidence intervals for through-year values. Statistical analyses were performed using AV Bio-statistics 4.8 (available at http://web.telia.com/~u25601709/avbs/) and SPSS 15.0.0 (SPSS Inc, USA).

Results

PRINCIPAL COMPONENT ANALYSIS

The four principal components (PC) having eigenvalues over 1 explained 70.8% of the total variance in immune parameters and divided the individual immune parameters into four functional categories (Table 2). The first PC (IC1) was positively related to the total number of leukocytes and relative count of lymphocytes, and correspondingly negatively related to the relative count of thrombocytes (Table 2). The second PC (IC2) related most clearly to phagocytes and

migration activity of head kidney phagocytes, but also to the IgM concentration. The third PC (IC3) was indicative of the presence of phagocytotic cells in the circulation, i.e. the relative counts of granulocytes and macrophages. The fourth PC was dominated by the spleen somatic index (Table 2).

**Seasonal Changes and Sex-Differences**

Since the seasonal changes in immune function have been reported in Kortet et al. (2003a), we do not report details about the individual immune parameters here. All principal components of immunity (IC1-IC4) varied seasonally (ANOVA, $F_{4,112} \geq 8.1$, $P < 0.01$, Fig. 1a–d). All but IC2 also differed between sexes (ANOVA, $F_{1,112} \geq 6.0$, $P < 0.02$). Furthermore, the interaction between sex and season was significant for IC1 and IC3 (ANOVA, $F_{4,112} \geq 3.9$, $P < 0.01$).

**Table 2.** Component score matrix of the three principal components of immune function. Varimax rotation with Kaiser normalization was used, and only eigenvalues over 1 are considered. The most important parameters contributing to the principal components are in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Score coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.92</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>-0.95</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>-0.06</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.04</td>
</tr>
<tr>
<td>Spleen-somatic index</td>
<td>0.18</td>
</tr>
<tr>
<td>Total leukocyte count</td>
<td>0.41</td>
</tr>
<tr>
<td>Chemiluminescence count</td>
<td>-0.14</td>
</tr>
<tr>
<td>IgM concentration</td>
<td>0.25</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>-0.04</td>
</tr>
<tr>
<td>Cumulative $R^2$</td>
<td>24.45</td>
</tr>
</tbody>
</table>

Fig. 1. **(a–d)** Seasonal variation in the four principal components of immune function (See Table 2 for description for PC: s). **(e–h)** Seasonal variation in the proportion of dead *Rhipidocotyle campanula* parasites and load of *R. campanula*, *R. fennica*, and *Raphidascaris acus*. Filled circles represent males and open boxes represent females. Error bars represent standard error of mean.

Table 3. Correlations between testosterone, oestradiol, the four principal components of immune function (Table 1), proportion of killed Rhipidocotyle campanula and load of R. campanula, R. fennica and Raphidascaris acus. In the upper right diagonal there are the Pearson's correlation over all periods and both sexes, and in the lower left diagonal there are the meta-analytically combined 95% confidence intervals for correlations calculated within each period and sex. N.S. is for not significant at 0.05 level

<table>
<thead>
<tr>
<th></th>
<th>Testosterone</th>
<th>Oestradiol</th>
<th>IC1</th>
<th>IC2</th>
<th>IC3</th>
<th>IC4</th>
<th>Dead R. camp. load</th>
<th>R. camp. load</th>
<th>R. fennica load</th>
<th>R. acus load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>1</td>
<td>0.391***</td>
<td>n.s.</td>
<td>0.393***</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>na.</td>
<td>1</td>
<td>−0.186*</td>
<td>0.201*</td>
<td>−0.211*</td>
<td>−0.202*</td>
<td>n.s.</td>
<td>0.273**</td>
<td>0.268**</td>
<td>n.s.</td>
</tr>
<tr>
<td>IC1</td>
<td>n.s.</td>
<td>n.s.</td>
<td>1</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>IC2</td>
<td>0.00−0.40</td>
<td>n.s.</td>
<td>na.</td>
<td>1</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>IC3</td>
<td>n.s.</td>
<td>n.s.</td>
<td>na.</td>
<td>na.</td>
<td>1</td>
<td>n.s.</td>
<td>0.342***</td>
<td>n.s.</td>
<td>n.s.</td>
<td>−0.183*</td>
</tr>
<tr>
<td>IC4</td>
<td>n.s.</td>
<td>n.s.</td>
<td>na.</td>
<td>na.</td>
<td>na.</td>
<td>1</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Dead R. camp.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>−0.54−0.11</td>
<td>0.01−0.45</td>
<td>n.s.</td>
<td>n.s.</td>
<td>l</td>
<td>n.s.</td>
<td>−0.275**</td>
<td>n.s.</td>
</tr>
<tr>
<td>R. campanula load</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>na.</td>
<td>l</td>
<td>0.517***</td>
<td>0.182*</td>
</tr>
<tr>
<td>R. fennica load</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>na.</td>
<td>l</td>
<td>n.s.</td>
<td>l</td>
</tr>
<tr>
<td>R. acus load</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>na.</td>
<td>na.</td>
<td>na.</td>
<td>l</td>
</tr>
</tbody>
</table>

*P < 0.05, **0.05 < P < 0.01, ***P < 0.001.

The proportion of dead R. campanula varied between seasons (ANCOVA, $F_{4,108} = 4.5$, $P = 0.002$) and by the interaction term season × sex ($F_{4,108} = 2.7$, $P = 0.032$, Fig. 1e). The total load of R. campanula did not vary seasonally ($F_{4,130} = 1.3$, $P = 0.280$), but was higher in females than in males ($F_{1,130} = 5.2$, $P = 0.02$, Fig. 1f). The interaction term was not significant in this case. Also the other Rhipidocotyle species, R. fennica was more abundant in females than in males ($F_{1,130} = 6.1$, $P = 0.015$), and did not vary between the seasons even in the interaction with sex (Fig. 1g). Raphidascaris acus was in general more abundant in females than in males ($F_{1,130} = 7.3$, $P = 0.008$, Fig. 1h), but also varied between seasons ($F_{4,130} = 2.7$, $P = 0.034$), and in the interaction between sex and season ($F_{4,130} = 2.9$, $P = 0.025$, Fig. 1h). Loads of other parasite species were not studied for seasonal changes or sex differences since their abundances were rather low (Table 1), and these aspects were not the focus of the study.

**Immune function and parasite loads**

IC3 correlated positively with the proportion of killed Rhipidocotyle campanula parasites and negatively with the load of Raphidascaris acus in the pooled samples (Table 3). Measures of immune function were mainly positively correlated with the load of several parasites when analysed using Spearman’s rank correlation in pooled samples (Table 4).

When the samples and sexes were analysed separately, and the individual correlation coefficients were combined meta-analytically, IC1 was found to correlate negatively with the proportion of dead R. campanula, whereas the IC2 correlated positively with it (Table 3). None of the parasite loads were significantly related to the measures of immunity (Table 3).

**Steroids and immune function**

Correlations of testosterone and oestradiol with individual immune measures are reported in Vainikka et al. (2004a). Plasma testosterone concentration correlated positively with the IC2 both when the samples and sexes were pooled, and in meta-analysed correlation based on seasonal correlations (Table 3). Concentration of oestradiol correlated negatively with all immune components, except for the positive correlation with IC2 in the pooled samples (Table 3). However, concentration of oestradiol was not correlated with any of the measures of immunity when the season effect was removed by meta-analysing the individual correlations (Table 3).

**Steroids and parasite loads**

Plasma oestradiol concentration correlated positively with the total load of R. campanula and R. fennici parasites in the pooled samples (Table 3). In non-parametric analyses, testosterone correlated negatively with the load of monogenean parasites on gills when the samples and sexes were pooled (Table 4). Oestradiol was negatively correlated with the loads of ectoprotozoa and Monogenea (Table 4). However, the concentrations of sex hormones were not related to the load of parasites in the meta-analytical results (Table 3).

**Epidermal papillomatosis, immune function and steroids**

Logistic regression with a backward model selection procedure starting from the model including sex (as factor), period (as factor), fish length, testosterone, oestradiol, IC1, IC2, IC3, IC4, and interactions testosterone × sex, oestradiol × sex was used to examine factors contributing to the probability of being diseased by the epidermal papillomatosis. The final model included the parameters; sampling period, length, oestradiol, testosterone and oestradiol × sex interaction but none of the immune measures. The final model explained 65.3% of the total variance and predicted 89.5% of the fish to the correct health/diseased class. Testosterone had a strong positive effect (B = 0.94) and oestradiol a negative effect (B = −0.66, in interaction with sex, B = −0.70) on the occurrence of papillomatosis.
Table 4. Spearman's rank correlations of testosterone, oestradiol and the four principal components of immune function (Table 2) with the load of several parasites and the proportion of dead Raphidascaris acus parasites of all R. acus individuals over both sexes and all periods. The proportion was defined only for fish that had at least three parasite individuals.

<table>
<thead>
<tr>
<th>Species &amp; location</th>
<th>Testosterone</th>
<th>Oestradiol</th>
<th>IC1</th>
<th>IC2</th>
<th>IC3</th>
<th>IC4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ectoprotozoa</td>
<td>R 0.14</td>
<td>-0.19</td>
<td>-0.17</td>
<td>0.02</td>
<td>0.27</td>
<td>0.02</td>
</tr>
<tr>
<td>Gills and epidermis</td>
<td>P 0.096</td>
<td>0.027</td>
<td>0.054</td>
<td>0.864</td>
<td>0.002</td>
<td>0.809</td>
</tr>
<tr>
<td>N 144</td>
<td>141</td>
<td>123</td>
<td>123</td>
<td>123</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Monogenea total</td>
<td>R -0.25</td>
<td>-0.24</td>
<td>-0.02</td>
<td>0.01</td>
<td>0.38</td>
<td>-0.01</td>
</tr>
<tr>
<td>Gills</td>
<td>P 0.003</td>
<td>0.005</td>
<td>0.833</td>
<td>0.866</td>
<td>&lt;0.001</td>
<td>0.953</td>
</tr>
<tr>
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<td>123</td>
<td>123</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Ergasilus sieboldi</td>
<td>R -0.11</td>
<td>-0.02</td>
<td>0.02</td>
<td>-0.15</td>
<td>0.12</td>
<td>-0.06</td>
</tr>
<tr>
<td>Gills</td>
<td>P 0.176</td>
<td>0.850</td>
<td>0.838</td>
<td>0.092</td>
<td>0.171</td>
<td>0.494</td>
</tr>
<tr>
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<td>123</td>
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</tr>
<tr>
<td>Myxobolus sp.</td>
<td>R 0.16</td>
<td>0.10</td>
<td>-0.13</td>
<td>0.14</td>
<td>0.21</td>
<td>-0.10</td>
</tr>
<tr>
<td>Gills</td>
<td>P 0.054</td>
<td>0.242</td>
<td>0.163</td>
<td>0.132</td>
<td>0.022</td>
<td>0.258</td>
</tr>
<tr>
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<td>141</td>
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<td>123</td>
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<td>123</td>
<td></td>
</tr>
<tr>
<td>Glochidia total</td>
<td>R 0.12</td>
<td>0.02</td>
<td>-0.10</td>
<td>0.18</td>
<td>0.20</td>
<td>0.19</td>
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<tr>
<td>Gills</td>
<td>P 0.161</td>
<td>0.826</td>
<td>0.278</td>
<td>0.046</td>
<td>0.025</td>
<td>0.038</td>
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</tr>
<tr>
<td>Endoproteozoa</td>
<td>R 0.13</td>
<td>0.06</td>
<td>-0.20</td>
<td>0.07</td>
<td>0.00</td>
<td>0.16</td>
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<tr>
<td>Fins</td>
<td>P 0.128</td>
<td>0.467</td>
<td>0.029</td>
<td>0.420</td>
<td>0.970</td>
<td>0.074</td>
</tr>
<tr>
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<td>123</td>
<td>123</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Neovergasilus japonicus</td>
<td>R 0.13</td>
<td>0.09</td>
<td>0.05</td>
<td>0.18</td>
<td>-0.02</td>
<td>-0.15</td>
</tr>
<tr>
<td>Fins</td>
<td>P 0.133</td>
<td>0.263</td>
<td>0.620</td>
<td>0.047</td>
<td>0.787</td>
<td>0.106</td>
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<td></td>
</tr>
<tr>
<td>Diplostomum sp.</td>
<td>R -0.13</td>
<td>-0.16</td>
<td>-0.03</td>
<td>0.02</td>
<td>-0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Eye</td>
<td>P 0.128</td>
<td>0.063</td>
<td>0.784</td>
<td>0.820</td>
<td>0.920</td>
<td>0.877</td>
</tr>
<tr>
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<td>123</td>
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<td></td>
</tr>
<tr>
<td>Tylodelphys clavata</td>
<td>R -0.01</td>
<td>0.11</td>
<td>0.20</td>
<td>-0.01</td>
<td>-0.04</td>
<td>-0.23</td>
</tr>
<tr>
<td>Eye</td>
<td>P 0.862</td>
<td>0.207</td>
<td>0.030</td>
<td>0.884</td>
<td>0.693</td>
<td>0.011</td>
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<tr>
<td>Prop. of dead R. acus</td>
<td>R 0.07</td>
<td>0.04</td>
<td>0.02</td>
<td>-0.06</td>
<td>-0.05</td>
<td>-0.19</td>
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<tr>
<td>Liver</td>
<td>P 0.564</td>
<td>0.734</td>
<td>0.855</td>
<td>0.661</td>
<td>0.726</td>
<td>0.127</td>
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<td>N 77</td>
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</tbody>
</table>

Discussion

We were able to divide several measures of immune function of roach to four functional categories by principal component analysis: (1) total leukocyte counts dominated by lymphocytes, (2) function of phagocytes including chemotaxis and oxidative burst, and IgM antibody concentration, (3) proportion of phagocytic cells in the blood, and (4) the relative size of the spleen. Our results show that the functional qualities of phagocytes (i.e. respiratory burst and chemotaxis) relate to the proportion of dead R. campanula parasites, potentially killed by host, in a wild fish species. Similarly, our results suggest that the measurement of IgM antibody concentration is positively associated to the ability of the fish to control parasites. In contrast, the (IC1) relative proportion of lymphocytes and count of leukocytes was negatively, and relative count of thrombocytes positively associated with the proportion of dead R. campanula. Previously, Vainikka et al. (2005) used a similar approach for tench (Tinca tinca) and also demonstrated divergent associations between the head kidney and blood phagocytes. The fourth immune component did not relate to the count of parasites, or proportion of dead parasites, which suggests that the relative size of the spleen may be a poor indicator of immune function in roach, and not related to the more direct measures of immunity (c.f. Vainikka et al. 2005).

The observed positive correlations between immune measures and parasite loads suggest that many measures of immune function may be up-regulated in response to increased parasite loads. However, the causal relationships are difficult and sometimes impossible to confirm in correlative studies. As detected in another cyprinid, bream (Abramis brama L.), allelic composition of major histocompatibility (MHC) genes can affect the occurrence of certain parasites and even the size of the spleen (Ottová et al. 2007). Therefore, experimental studies are urgently needed to link the MCH to immune function and susceptibility to parasites both in cyprinids and in general. In our study, seasonal covariation probably reflected to the observed correlations in the pooled data, but also affected the formation of the immune components. However, seasonal covariation between the parameters of immunity likely indicates a common functional category rather than a fully artificial collinear seasonal fluctuation. Therefore, our results suggest that relatively simple measures of immune function, such as those included in the IC2, can be biologically meaningful; here roach with high immune component two (IC2) values had efficiently eliminated R. campanula parasites in their body.
The observed relationship between the IC2 and the proportion of dead *R. campanula* parasites is interesting, particularly in light of the following. Firstly, *R. campanula* has been suggested to be harmful to roach (Baturó 1977), and it is prevalent and abundant in the current study population, as well as in many other roach populations (Taskinen & Kortet 2002). Secondly, breeding tubercles of roach are known to convey information about the proportion of dead *R. campanula* (Taskinen & Kortet 2002); therefore sexual ornaments of roach may signal good functional immunocompetence. Roach are known to produce effective specific antibodies against another *Rhipidocotyle* species; *R. fennica* that occurs predominantly on fins (Aaltonen et al. 1997). Thus, it is likely that the resistance against *R. campanula* is also mediated by specific antibodies (see also Aaltonen et al. 1994). The positive association between the concentration of antibodies and functional measures of phagocytes was also expected since phagocytosis is strongly enhanced by specific antibodies (Verho et al. 2005). However, the proportion of dead parasites observed on individuals captured in the wild is susceptible to ecological factors, and may not represent a direct measure of immunocompetence. Uneven exposure to these parasites might bias also the proportions especially over time, as older individuals might accumulate more dead individuals than young fish. The clearance time of dead parasite specimens is not known. However, at population level there are no reasons to expect biasing effects in addition to large variation in the abundance of these parasites.

Granulocytes and macrophages form the most important cellular first-line defence in fish (Secombes & Fletcher 1992). Granulocytes are able to migrate towards chemotactic stimulus and kill ingested pathogens by destructive enzymes and oxygen radicals in the reaction known as respiratory burst. Increased number of leukocytes, especially granulocytes, is a common consequence of infection (e.g. Ellis 1999; Pulsford et al. 1995). Thus, the observed positive correlation between parasite loads and the relative proportion of phagocytic cells in blood in the pooled materials was expected. Also, it was found that the functional characteristics of these cells were positively related to the proportion of dead *R. campanula* parasites, suggesting that the chemiluminescence method assaying the respiratory burst may be a valuable tool to estimate functional immunocompetence. Supporting the importance of chemiluminescence in resisting parasites, the scuticociliate parasite *Uronema marinnum* was found to inhibit the respiratory burst response of olive flounder *Paralichthys olivaceus* as a way to increase virulence (Kwon et al. 2002). Moreover, interspecies comparison between coho salmon (*Oncorhynchus kisutch*), rainbow trout (*O. mykiss*) and common carp (*Cyprinus carpio*) suggests that the differences in the chemiluminescence response between these species related to their resistance against *Renibacterium salmoninarum* (Sakai et al. 1996).

Spleen size is commonly used as an indicator of immunocompetence (e.g. Saino, Calza & Møller 1997; Šimková et al. 2008), is often sexually dimorphic (Møller, Soré & Erritzoe 1998; Kortet et al. 2003b), and correlates positively with the abundance of parasites in a within species comparison of cyprinid fishes (Šimková et al. 2008). However in our analysis on roach, the relative spleen size did not relate to the other more direct measures of immune function and the IC4, most strongly affected by the spleen size, was not related to the parasite counts or proportions of dead parasites. Although the size of the spleen may increase following an infection (e.g. Lefebvre et al. 2004), our analysis suggests that spleen size might not represent immunocompetence of roach and should be interpreted with caution in immunological studies.

Several aspects of immune function show seasonal changes as a response to challenging time periods such as winter, and physiological demands such as reproduction (Kortet & Vainikka 2008). Reproduction has been shown to impair immune function and increase parasitism (e.g. in Arctic char, *Salvelinus alpinus* (Skarstein et al. 2001). Breeding-related immunosuppression is thought to result from a high breeding-time concentration of androgens (Aida 1988; Folstad & Karter 1992; Hou, Suzuki & Aida 1999a,b; Muñoz et al. 2000), although changes in water temperature and in the concentration of corticosteroids may also play a role in explaining this annual variation (Zapata, Varas & Torroba 1992; Collazos, Ortega & Barriga 1994; Alcorn, Murray & Pascho 2002). The seasonal variation in the individual measures of immunity we used has been described previously by Kortet et al. (2003a). However, the present combined measures of immunity showed somewhat different patterns: changes in IC1 suggested breeding-related suppression in male roach, whereas both IC2 and IC3 showed high values during the spawning period in spring. These changes may reflect the direct effects of immunosuppressive hormones but also indirect effects of increased parasite loads at the time of breeding. Therefore, experimental studies are needed to address the causal factors behind these seasonal changes. Interestingly, size-adjusted parasite loads were often higher in females than in males, suggesting that the relatively high reproductive effort of females in terms of egg production may contribute to the observed gender differences more than the potential androgen-dependent immunosuppression.

The occurrence of epidermal papillomatosis has been shown to be highly season-dependent, peaking during the spawning period, and being more common in males than in females (Kortet, Vainikka & Taskinen 2002). The occurrence of the disease has been suggested to be related to the concentrations of both sex (Kortet et al. 2003b) and stress hormones (Vainikka, Kortet & Taskinen 2004b). In this study, we confirmed that high testosterone levels contribute to the development of the disease. Interestingly, it seemed that high oestadiol levels prevented outbreak; which might explain the clear sex difference in the disease occurrence. However, in this study we did not determine cortisol levels, and the relationship between sex hormones and the epidermal papillomatosis could have been caused by a correlated stress response (Vainikka et al. 2004b).

Although immunosuppressive effects of testosterone were thought to be well understood, at least in mammals (Grossman 1985); the recent meta-analysis of studies testing the immunocompetence-handicap hypothesis (Folstad & Karter 2004)
1992) by Roberts, Buchanan & Evans (2004) suggests that in many species testosterone may increase susceptibility to ectoparasites, but direct effects on immune function may not exist. For example, in red-winged blackbirds (Agelaius phoeniceus) a clear absence of a response on immune function by testosterone has been reported (Hasselquist et al. 1999). In contrast, many studies of lizards clearly support the immunosuppressivity of testosterone (e.g. Saad, Khalee & El Ridi 1990; Kluowski & Nelson 2001). At the population level, the trade-offs predicted by life-history theory might be difficult to observe because individuals of better overall quality may show superior performance in several traits, even if trade-offs operate between the traits at individual level (Van Noordwijk & de Jong 1986). This might have contributed to our lack of detection between the traits at individual level (Van Noordwijk & de Jong 1986).

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