JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2011.02273.x

Bateman's principle and immunity in a sex-role reversed pipefish

O. ROTH*, J. P. SCHARSACK†, I. KELLER* & T. B. H. REUSCH*

*Leibniz Institute for Marine Sciences, IFM-GEOMAR, Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg, Kiel, Germany
†Institute for Evolution and Biodiversity, University of Münster, Hüfferstrasse, Münster, Germany

Keywords:

Bateman's principle;
immune defence;
immunocompetence;
pipefish;
sex;
sex-role reversal;
sexual dimorphism.

Abstract

In diverse animal species, from insects to mammals, females display a more efficient immune defence than males. Bateman's principle posits that males maximize their fitness by increasing mating frequency whereas females gain fitness benefits by maximizing their lifespan. As a longer lifespan requires a more efficient immune system, these implications of Bateman's principle may explain widespread immune dimorphism among animals. Because in most extant animals, the provisioning of eggs and a higher parental investment are attributes of the female sex, sex-role reversed species provide a unique opportunity to assess whether or not immune dimorphism depends on life history and not on sex *per se*. In the broad-nosed pipefish *Syngnathus typhle*, males brood and nourish the eggs in a ventral pouch and thus invest more into reproduction than females. We found males to have a more active immune response both in field data from four populations and also in an experiment under controlled laboratory conditions. This applied to different measures of immunocompetence using innate as well as adaptive immune system traits. We further determined the specificity of immune response initiation after a fully factorial primary and secondary exposure to a common marine pathogen *Vibrio* spp. Males not only had a more active but also a more specific immune defence than females. Our results thus indeed suggest that the sex that invests more into the offspring has the stronger immune defence.

Introduction

The origin of males and females can be traced back to the evolution of anisogamy that defines males as the sex producing small gametes (sperm) whereas females provide large ones (eggs) (Stearns, 1987). The selective advantage for anisogamy is still under debate, ranging from sexual conflict (Parker *et al.*, 1972) to sexual cooperation (Iyer & Roughgarden, 2008).

However, contemporary selection pressures on both sexes vary (Klein, 2000), causing the evolution of differing life history strategies for males and females. As females can only produce a limited amount of eggs per unit time (Williams, 1966), female fitness is positively correlated with longevity. Males, on the other hand, can mate promiscuously to increase their chances to fertilize the limited number of existing eggs. Male fitness is thus maximized by increasing mating rates and relies on immediately available resources that can be spent whenever potential mating partners are abundant (Bateman's principle) (Bateman, 1948; Trivers, 1972; Clutton-Brock, 1988). Under this scenario, intra-sexual selection for the male sex is intensified (Bateman, 1948). Assuming limited resources and a resulting allocation trade-off, we expect reduced investment, for example, into immunocompetence, evident in less efficient defence against potential parasite and pathogen attacks (Zuk & Stoehr, 2002).

In contrast, females are typically under selection to maximize the length of their reproductive period, which can be realized by lengthening the lifespan. This in turn requires costly investment into immune defence. These implications from Bateman's principle could thus explain the widespread sexual dimorphism in immune defence among animals (Rolff, 2002).

Corresponence: Olivia Roth, Leibniz Institute for Marine Sciences, IFM-GEOMAR, Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg 20, D-24105 Kiel, Germany.
Tel.: +49 431 600 4557; fax: +49 431 600 4553;
e-mail: oroth@ifm-geomar.de

Empirical data overwhelmingly support the notion that females have a more efficient immune response and parasite defence than males across the animal kingdom (e.g. Kurtz *et al.*, 2000; Roth *et al.*, 2008; Schuurs & Verheul, 1990; Siva-Jothy, 2000; Zuk & Mc Kean, 1996). The mechanism for this pattern is less clear and still under debate. The most prominent explanation goes back to honest signalling of good genes by displaying sexual signals at the cost of reduced immune defence (Hamilton & Zuk, 1982; Folstad & Karter, 1992). A high level of testosterone was proposed to induce the expression of secondary sexual signals whilst simultaneously assuming that testosterone has an immunosuppressive effect (Folstad & Karter, 1992). However, this mechanism cannot be universal as invertebrates lacking testosterone also display the predicted difference in immune investment (Braude *et al.*, 1999; Peters, 2000; Sheridian *et al.*, 2000; Bilbo & Nelson, 2001; Kurtz & Sauer, 2001; McKean & Nunney, 2001). In addition, a meta-analysis failed to support the notion that testosterone suppresses immunity (Roberts *et al.*, 2004). Consequently, Sheldon & Verhulst (1996) argued that a resource allocation trade-off accounts for sexual dimorphism in immunity. As only a certain pool of resources is available to serve all life history traits, investing more into ornamentation may result in a trade-off such that not enough resources are available for an efficient immune defence (Sheldon & Verhulst, 1996).

Because in animals with conventional sex roles, the provisioning of eggs and parental care are both inseparable attributes of the female sex, sex-role reversed species provide unique opportunities to demonstrate whether or not immune dimorphism depends on life history or on sex per se. In general, sex-role reversed species, where competition-driven sexual selection is higher in females than in males, can serve as valuable tool for testing hypotheses about sexual selection (Williams, 1975). Here, the phylogenetically much older anisogamy of sexual products still defines males and females while the life history strategy such as mate choice and investment into offspring is inverted (Clutton-Brock & Vincent, 1991). The Bateman gradient that suggests selection to be stronger on males than on females because of the greater pay-off by mating (Andersson, 1994; Arnold, 1994) has been demonstrated to be inverted in the sex-role reversed pipefish *Syngnathus typhle*. This implies that stronger sexual selection acts on females (Jones *et al.*, 2000, 2005).

We expect that also Bateman's principle is reversed in a sex-role reversed pipefish. Specifically, we predict that sexual dimorphism in immunity manifests itself as stronger immune investment and immunocompetence in the males compared to females, which would support a role for different life history constraints as ultimate reason for such dimorphism (Rolff, 2002).

In the present study, differences of the pipefish immune system among the sexes were investigated on the cellular (leucocyte) and the humoral (blood plasma) level. Pipefish leucocytes were isolated from the head kidney, the major immune organ in bony fish (Tort & Mackenzie, 2003), where leucocytes are produced and proliferate (comparable to bone marrow and lymphatic organs of mammals). Furthermore, leucocytes activated in the periphery by pathogenic challenge migrate to the head kidney to induce activation of leucocyte subsets (lymph node of mammals). Thus, the absolute number of lymphocytes and monocytes but also the composition (number of lymphocytes versus monocytes) and activity of head kidney leucocytes (HKL) is representative for the actual cellular immune status of a fish. Humoral immunity in bony fish depends on the antimicrobial activity of proteins (e.g. lysozyme, complement and antibodies), which opsonize and destroy invading pathogens. Although few details are known on the immune system of Syngnathids (but see Matsunaga & Rahman, 1998), an analysis of head kidney leucocytes and humoral immunity provides useful measures to investigate individual differences in immunity of pipefish.

We first examined the sexual dimorphism in immune responses in *S. typhle* using field data of four natural pipefish populations. Second, sex differences in the immune response were assessed under controlled conditions in an experiment by priming and activating the immune response of *S. typhle* with heat-killed *Vibrio* spp bacteria, a common pathogen in the marine realm. The present study is, to the best of our knowledge, the first description of activity of the immune system in a sex-role reversed species.

Materials and methods

The host–parasite system

The broad-nosed pipefish *Syngnathus typhle* is widespread in macroalgal and seagrass beds along European coastlines (Wilson & Veragut, 2010). During copulation, females transfer eggs to the brood pouch located on the ventral surface of the male. Here, eggs are fertilized and carried by the male until embryos are born several weeks later (Berglund *et al.*, 1986a,b). Males thus have 100% paternity confidence (Jones & Avise, 1997). *S. typhle* is sex role reversed as males are much choosier than females, ultimately driven by much higher investment into the progeny (Berglund *et al.*, 1986b). Accordingly, intra-sexual competition in females is stronger, and they display nuptial ornaments to attract males (Berglund *et al.*, 1986a, 2005, 2006). The number of eggs produced by females by far exceeds the available male brooding capacity (Berglund *et al.*, 1989; Berglund & Rosenqvist, 1993); hence, Bateman's principle is reversed in *S. typhle* (Jones *et al.*, 2000, 2005).

Vibriosis is a common disease in aquaculture and aquaria fishes caused by the bacteria genus *Vibrio* (Hjeltnes & Roberts, 1993). *Vibrio* seems to be ubiquitous

in the aquatic environment and is claimed to be responsible for substantial negative impacts on fisheries (Austin & Austin, 1999). *Vibrio* species are found in high density on various marine organisms (Heidelberg *et al.*, 2002; Rosenberg & Ben-Hain, 2002; Sawabe *et al.*, 2003; Vandenberghe *et al.*, 2003), including Syngnathids (Alcaide *et al.*, 2001; Balcazar *et al.*, 2010a,b). The *Vibrio* strains used here were isolated during a parallel study across Europe using ten pipefish per location and plating samples from liver, head kidney, stomach and gill on *Vibrio*-sensitive agar.

Pipefish population and immunocompetence measurements

In May-June 2010, pipefish (*Syngnathus typhle*) from four populations were collected by snorkelling using hand nets and brought to the aquaria facilities at the IFM-GEOMAR in Kiel (Germany): Population 1: Venice Lagoon (Italy, N 45°25.20′; E 12°27.85′), Population 2: Wackerballig (Germany, N 54°75,57′; E 9°87,66′); Population 3: Lemvig (Denmark, N 56°56,30′; E 8°29.61′); and Population 4: Fiskebäckskil (Sweden, N 58°24.80′; E 11°44.62′). At all four locations also *Vibrio* prevalence was measured by plating organs of ten fish per location on *Vibrio*-sensitive TCBS agar. *Vibrio* prevalence in the field varied between 20% in Sweden, 45% in Germany and 65% in Denmark and Italy.

Animals were kept for 1–3 weeks in large tanks (2–4 animals per aquarium, a total of 16 aquaria of 80l) in a recirculation system at 18 °C with a 16 : 8 h light : dark regime (summer conditions) at the salinity of the habitat of origin (15–30 psu). Baltic Sea water was filtered (20 μm), treated with UV-B light and exposed to ozone (redox potential: 350) to reduce ambient pathogens and parasites. The salinity was adapted to original salinities using artificial sea salt (Instant Ocean, Aquarium System, France). Animals were kept in low densities to avoid stressful conditions. In addition, animals were separated by sex to assure that animals did not reproduce. Thus, at the day of the immune measurements, even though all animals were mature, males were not carrying eggs in their brood pouch, as pregnancy could affect the immune response of male pipefish. Beginning of July, the animals (separated per sex and location) were anaesthetized with MS 222 (Tricaine, 0.5 g L^{-1}; Sigma, Munich, Germany) and killed by incision of the brain. Pipefish weight (to the nearest 0.1 mg) and length (from the snout to the base of the tail, to the nearest mm) were determined. The body cavity was opened, and the head kidney was removed for immunological assays.

Vibrio immunization experiment

In late June, mature but nonpregnant pipefish were collected by snorkelling in a seagras meadow (*Zostera marina*) in Lemvig (Denmark) using hand nets and brought to the aquaria facilities in Kiel. After 2 weeks of acclimatization, fish were immune primed. To this end, the *Vibrio* strain I11Ma2 (isolated from the gut of an Italian pipefish in May 2010, phylogenetically most closely related to *Vibrio gigantis*) were grown in an overnight culture in medium 101 (5 g Peptone, 3 g Meat extract, 30 g NaCL, 1000 mL distilled water) at 25 °C. Upon centrifugation (10′ at 1600 r.p.m.), the bacteria pellet was resuspended in 1 mL PBS (phosphate-buffered saline pH 7). Bacteria were heat-killed (60 min at 65 °C), and the concentration was adjusted to 10^9 bacteria cells mL^{-1}. The bacteria were heat-killed to avoid potentially confounding fitness reducing effects because of an infection, such as only activation of immune defence upon *Vibrio* exposure has been measured in this experiment.

Fish (n = 30 males and females, respectively) were injected with 50 μL bacteria suspension into their body cavity [intraperitoneal (IP) injection with 5×10^7 bacteria cells]. Control fish were sham-injected with 50 μL PBS (Caipang *et al.*, 2008). The fish were kept in 16 tanks (four tanks per gender and treatment) in a recirculating system and fed daily with mysid shrimps and copepods (*Cyclops* sp.). Aquaria temperature was kept at 18 °C with a 16 : 8h light: dark regime (summer conditions). After 18 days, 26 of the *Vibrio*-injected animals were still alive, 13 males and 13 females. Of the sham-injected fish, 23 were still alive, 14 females and 9 males. Remaining fish were fully factorial and randomly assigned to either a secondary exposure to heat-killed *Vibrio* or a secondary injection with PBS resulting in eight treatment groups and six to seven fish per treatment group and sex, whereas male control group only consisted of four replicates. The treatment groups were as follows: females exposed to *Vibrio* during first and secondary exposure fV+V+; males exposed to *Vibrio* at first and second exposure mV+V+ and so on: fV−V+, mV−V+; fV+V−, mV+V−; fV−V−, mV−V−. Six days after the secondary exposure, animals were anaesthetized with MS 222 (Tricaine, 0.5g L^{-1}, Sigma) and killed by incision of the brain.

Blood was drawn from the caudal vein into heparin-coated haematocrit capillaries. Supernatant plasma was collected after centrifugation and immediately frozen at −20 °C. Pipefish weight (to the nearest 0.1 mg) and length (from the snout to the base of the tail, to the nearest mm) were determined. The Body cavity was opened, and the head kidney was removed for immunological assays. Animal experiments were performed in accordance with animal welfare legislation.

Immune assays

Isolation of head kidney leucocytes

Immunological assays were performed according to protocols developed for sticklebacks (Scharsack *et al.*, 2004, 2007a,b) with some modifications. For the isolation of leucocytes from pipefish head kidney, all steps

were performed on ice and only with refrigerated media and cooled centrifuges. Cell suspensions from head kidneys were prepared by forcing the tissues through a 40 μm nylon mesh screen. Isolated HKL were washed twice (4 °C, 10 min 550 *g*) with RPMI 1640 medium and resuspended in a final volume of 450 μL medium.

Flow cytometric analysis of pipefish leucocytes

Leucocyte subsets in HKL isolates were distinguished according to their light scatter profiles [forward scattered light (FSC) – cell size, side scattered light (SSC) – cell complexity] by means of flow cytometry (FACS-Calibur, Becton & Dickinson, Heidelberg, Germany). Lymphocytes (FSC/SSC_{low}) and number of monocytes (FSC/SSC^{high}) among HKL were counted. Absolute lymphocyte number, lymphocyte index (lymphocytes/mg body weight), absolute monocyte number, monocyte index (monocytes/mg body weight) and lymphocyte-to-monocyte ratio were calculated to estimate the cells of the adaptive (lymphocyte) versus the innate (monocyte) cellular immunity. Number of total viable HKL in individual samples was determined with exclusion of cellular debris (low scatter characteristics) and dead cells by propidium iodide stain (2 mg L^{-1}) with a flow cytometer to adjust HKL numbers equally across individual fish for subsequent immune assays.

Cell cycle analysis

Lymphocyte (B- and T-cell) proliferation is a significant part during an adaptive immune response. Accordingly, the proportion of lymphocytes in the synthesis (S) and mitosis (M) phase of the cell cycle can be used as a measure for the activity of the adaptive immune system. Here, the relative number of lymphocytes in the G_{0-1} phase, S Phase and G_{2-M} phase of the cell cycle was determined after DNA labelling with propidium iodide by means of flow cytometry (Scharsack *et al.*, 2007a,b). During the cell cycle in the G_{0-1} phase, cells have a single set of chromosomes and a constant content of total DNA. Cells starting to proliferate enter the S (synthesis) phase and are characterized by increasing amounts of DNA per cell. In the G_{2-M} phase, cells have completed DNA synthesis and are endowed with a double set of chromosomes (DNA) and start to divide. Proliferating cells in the G_{2-M} phase can be distinguished from G_{0-1} and S phase cells by their higher DNA content. For cell cycle analysis, head kidney leucocytes were fixed with ethanol (100 μL cell suspension in 200 μL ice cold Ethanol 98%) and stored at 4 °C. For analysis, cells were centrifuged (550 *g*, 10 min, 4 °C), and supernatant ethanol was removed. Propidium iodide (Sigma Aldrich, Munich, Germany) was added to a final concentration of 5 mg L^{-1}, and cells were incubated again for 10 min at room temperature. Individual samples were measured for three minutes or up to 30 000 events with a flow cytometer (Becton Dickinson FACSCalibur). Red fluorescence (propidium iodide) was measured in linear mode. Cellular debris (low scatter characteristics) and aggregated cells (high scatter characteristics) were subtracted for further evaluation. Doublet cells were subtracted from single cells as described by Wersto *et al.* (2001). Lymphocytes were identified according to their characteristic FSC/SSC profile. Frequencies of lymphocytes in G_{0-1}, S and G_{2-M} phase were acquired by DNA content analysis of red fluorescence intensity (propidium iodide labelling) of single cells from the lymphocyte gate.

Respiratory burst activity of head kidney leucocytes

As one of the most important effector mechanisms of cell-mediated innate immunity, the respiratory burst activity of HKL was quantified in a lucigenin-enhanced chemiluminescence assay (CL) modified after Scott & Klesius (1981), as described in Kurtz *et al.* (2004). In white 96-well flat-bottom microtitre plates, 70 μL of cell suspension (1×10^5 HKL $well^{-1}$) was added to 20 μL lucigenin solution (2.5 g L^{-1} PBS) and 60 μL RPMI 1640 medium. Phagocytosis and production of reactive oxygen species were initiated by the addition of 20 μL zymosan suspension (7.5 g L^{-1} PBS), and 20 μL PBS was added to the corresponding controls. Chemoluminescence owing to reaction of lucigenin with oxygen radicals was measured for 3 h at 20 °C with a microtitre plate luminometer (Tecan Infinite 200). Respiratory burst activity was expressed as total chemoluminescence (area under curve) produced by zymosan-stimulated cultures relative to controls.

Antimicrobial activity (only Vibrio immunization experiment)

To measure the antimicrobial activity against either the *Vibrio* strain animals were exposed to but also against novel *Vibrio* strains, three bacterial strains of our *Vibrio* cultures were chosen: I11Ma2, D3K3 and I2K3. The bacteria were grown in medium in an overnight culture, centrifuged at 1600 rpm, counted using a Thoma counting chamber and then diluted to a density of 10^9 cells mL^{-1}. Medium 101 with agar (7.5 g L^{-1} medium, 15 g NaCl) was autoclaved and cooled down to 40 °C. Upon addition of bacteria to a concentration of 10^6 cells mL^{-1}, medium and petri dishes were filled with 4ml bacterial medium. After polymerization, eight holes per petri dish received 2 μL fish plasma each, one hole with an antibiotic sample of tetracycline served as positive and a water samples as negative control. The petri dishes were incubated for 14 h at 25 °C. Subsequently, inhibition zones were measured with a calliper to the nearest 0.1 mm. On every plate, inhibition zone measures were standardized to the tetracycline inhibition zone to account for inter-dish variation.

Statistical analyses

The pipefish population and immunocompetence measurements were analysed with two-way ANOVAS with sex

and origin as fixed factors. Origin was not treated as a random factor as we were specifically interested in comparing these four populations. As response variables, we analysed the lymphocyte/monocyte ratio, absolute lymphocyte count, lymphocyte index (lymphocytes/mg body weight), absolute monocyte count, monocyte index (monocytes/mg body weight), cell cycle (G_{0-1} phase, S Phase and G_{2-M} phase) and respiratory burst. The random effect 'tank' was tested in an ANOVA. As the effect was clearly not significant ($P > 0.3$) in all tested factors, it was omitted from further analyses, for the sake of reduced risk to commit type II errors (Underwood, 1997).

The pipefish *Vibrio* immunization experiment was analysed with three-way ANOVAS taking sex, 1st exposure and 2nd exposure as fixed factors. Lymphocyte/monocyte ratio, absolute lymphocyte count, lymphocyte index (lymphocytes/mg body weight), absolute monocyte count, monocyte index (monocytes/mg body weight), cell cycle (G_{0-1} phase, S Phase and G_{2-M} phase), respiratory burst and antimicrobial activity (activity against I11Ma2, D3K3 and I2K3) were the response variables.

All data were tested for normal distribution using residuals. If normal distribution was not fulfilled, data were transformed accordingly.

Results

Pipefish population and immunocompetence measurements

Males had higher absolute lymphocyte numbers than females (female: 10043 ± 1290 (mean ± SE), males: 13937 ± 1712) (two-way ANOVA: F-model $_{7, 38}$; F origin$_{3, 38}$ = 9.176, $P < 0.0001$; F sex$_{1, 38}$ = 4.925, $P = 0.0322$; F origin x sex$_{3, 38}$ = 2.139, $P = 0.1104$). Also, the lymphocyte index (absolute number of lymphocytes/bodyweight in mg) was higher in males than in females (female: 3.80 ± 0.663 (mean standard error), males: 6.79 ± 1.311) (two-way ANOVA: F-model$_{7, 38}$; F origin$_{3, 38}$ = 3.171, $P = 0.0207$; F sex$_{1, 38}$ = 5.5773, $P = 0.0232$; F origin × sex$_{3, 38}$ = 1.2675, $P = 0.2985$). Animals from different populations varied in their absolute lymphocyte number (Denmark: 11667 ± 1504, Italy: 15977 ± 1548; Germany: 15544.5 ± 2382. Sweden: 3821 ± 1616) and in their lymphocyte index (Denmark: 5.88 ± 0.501, Italy: 3.64 ± 0.675; Germany: 8.43 ± 2.198. Sweden: 2.50 ± 1.907), especially Sweden had extremely low cell counts compared to all three other locations. Monocyte absolute numbers (two-way ANOVA: F-model $_{7, 38}$; F origin$_{3, 38}$ = 10.2998, $P < 0.0001$; F sex$_{1, 38}$ = 0.1598, $P = 0.6915$; F origin × sex$_{3, 38}$ = 2.3674, $P = 0.0851$) and monocyte index (two-way ANOVA: F-model $_{7, 38}$; F origin$_{3, 38}$ = 4.1892, $P < 0.0114$; F sex$_{1, 38}$ = 2.0723, $P = 0.1578$; F origin × sex$_{3, 38}$ = 2.0450, $P = 0.1230$) were consistent between males and females, but absolute monocyte number (Denmark: 24125 ± 1610, Italy: 19469 ± 2272; Germany: 24770 ± 1799. Sweden: 8260 ± 9635) as well as monocyte index varied among locations (Denmark: 13.59 ± 1.571, Italy: 4.77 ± 0.88; Germany: 12.37 ± 2.02. Sweden: 4.99 ± 3.66).

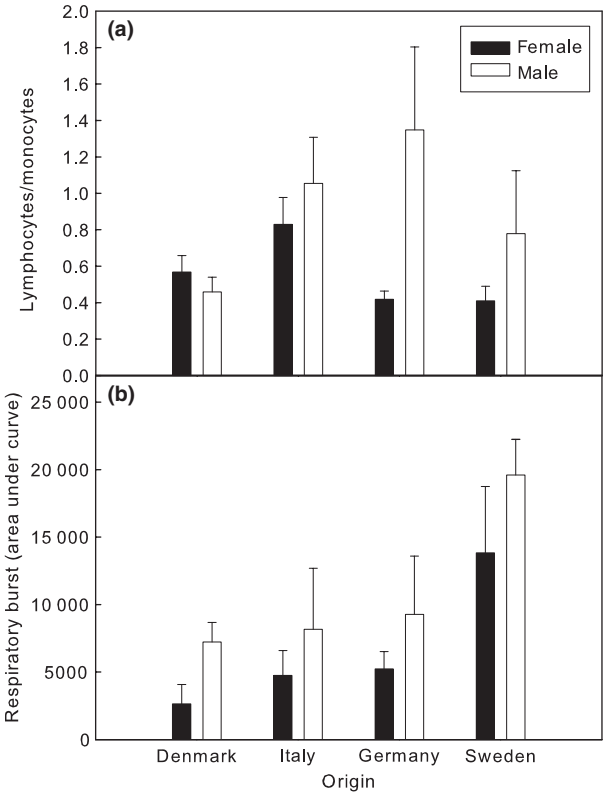

Fig. 1 Immune parameters of four different pipefish populations (Denmark, Italy, Germany and Sweden), displayed for females (black bars) and males (light bars). The lymphocyte-to-monocyte ratio (a) is an indicator for the proportion of immune cells of the adaptive immune system (lymphocytes) to the proportion of immune cells of the innate immune system (monocytes). Respiratory burst (b) shows the phagocytosis activity of monocytes. Bars show mean + SE.

Males had a higher proportion of cells from the adaptive immune system (lymphocyte-to-monocyte ratio) than females [female: 0.54 ± 0.054 (mean ± SE), males: 0.79 ± 0.135]. Animals from different populations varied in their lymphocyte-to-monocyte ratio. Whereas Italian pipefish had the highest lymphocyte-to-monocyte ratio, Denmark was the lowest, and Germany and Sweden were in between (Fig. 1a). The interaction of sex and origin had a significant effect of lymphocyte-to-monocyte ratio (two-way ANOVA: F-model $_{7, 38}$; F origin$_{3, 38}$ = 3.171, $P = 0.035$; F sex$_{1, 38}$ = 6512, $P = 0.015$; F origin × sex$_{3, 38}$ = 3.080, $P = 0.039$).

Males had a higher respiratory burst (phagocytosis activity) than females. Pipefish from the Swedish population had a higher respiratory burst than fish from all

other populations (Fig. 1b) (female: 6638.35 ± 1570; male: 9682.25 ± 1730) (two-way ANOVA: F-model $_{7, 36} = 2.713$; F origin$_{3, 36} = 5.423$, $P = 0.004$; F sex$_{1, 36} = 4.132$, $P = 0.049$; F origin × sex$_{3, 36} = 0.045$, $P = 0.987$).

Females had a slightly higher proportion of cells in the resting stage than males ($G_{0–1}$ phase) (female: 0.755 ± 0.008; male: 0.722 ± 0.008) (two-way ANOVA: F-model $_{7, 37} = 2.073$; F origin$_{3, 37} = 0.389$, $P = 0.761$; F sex$_{1, 37} = 4.237$, $P = 0.046$; F origin × sex$_{3, 37} = 1.641$, $P = 0.197$).

Vibrio immunization experiment

Males had a higher absolute lymphocyte count and a higher lymphocyte index than females. Absolute monocyte count but also monocyte index was not affected by sex (Table 1a–d, Fig. 2a, b). Males had a higher proportion of lymphocytes compared to females. Males that were twice exposed to *Vibrio* had the highest proportion of lymphocytes (Fig. 3a, Table 2a). A secondary exposure to *Vibrio* led to a higher lymphocyte-to-monocyte ratio. The significant three-way interaction of sex × 1st × 2nd exposure suggests that the two sexes react differently on the combination of 1st and 2nd exposure. Females had a lower phagocytosis activity than males. In addition, if males were treated with PBS upon secondary exposure, their phagocytosis activity was higher than for females regardless whether the latter were sham or *Vibrio* injected (Fig. 3b, Table 3). Males had a higher proportion of proliferating cells (S phase), suggesting a more active adaptive immune system (Fig. 4, Table 2b). Females had a higher antimicrobial activity against novel *Vibrio* strains they had not been exposed to during the experiment (D3K3, I2K3). However, if antimicrobial activity was measured against I11Ma2, the strain that pipefish were already exposed to, no sex difference was detectable. Interestingly, a significant three-way statistical interaction indicates that, depending on first vs. secondary exposure, males and females react differently to *Vibrio* (Fig. 5). This indicates that males catch up with the antimicrobial activity to female levels if an earlier exposure with the same strain occurred (Table 4). All measured immune responses classified into constitutive and inducible are again summarized according to their strength for males and females in Table 5.

Table 1 Analysis of the effects of sex and *Vibrio* priming vs. sham injection (during 1st or 2nd exp) on immune parameters using three-way factorial ANOVA. Response variables are lymphocyte index (absolute lymphocyte count/bodyweight in mg) (a), monocyte index (absolute monocyte count/bodyweight in mg) (b), absolute lymphocyte count (c) and absolute monocyte count (d). *$P < 0.05$.

ANOVA				d.f.
Model				7
Error				32
Total				39
	d.f.	Estimate	*t* ratio	*P*
(a) Lymphocyte Index				
Sex	1	−2.063	−2.05	0.048*
1st exp	1	−0.012	−0.01	0.990
Sex × 1st exp	1	−0.678	−0.68	0.504
2nd exp	1	−1.324	−1.32	0.197
Sex × 2nd exp	1	−0.891	−0.89	0.382
1st exp × 2nd exp	1	0.789	0.79	0.433
Sex × 1st exp × 2nd exp	1	0.512	0.51	0.614
(b) Monocyte Index				
Sex	1	−1.253	−1.00	0.323
1st exp	1	0.105	0.08	0.934
Sex × 1st exp	1	−2.390	−1.91	0.065
2nd exp	1	−0.615	−0.49	0.626
Sex × 2nd exp	1	−1.283	−1.03	0.312
1st exp × 2nd exp	1	1.246	1.00	0.326
Sex × 1st exp × 2nd exp	1	0.957	0.77	0.450
(c) Lymphocyte Count				
Sex	1	−1782.938	−3.80	< 0.001*
1st exp	1	−271.613	−0.58	0.567
Sex × 1st exp	1	445.987	0.95	0.350
2nd exp	1	−515.888	−1.10	0.280
Sex × 2nd exp	1	−750.488	−1.60	0.120
1st exp × 2nd exp	1	678.948	1.45	0.158
Sex × 1st exp × 2nd exp	1	423.938	0.90	0.374
(d) Monocyte Count				
Sex	1	−253.800	−0.25	0.801
1st exp	1	57.300	0.06	0.955
Sex × 1st exp	1	−969.300	−0.97	0.339
2nd exp	1	653.325	0.65	0.518
Sex × 2nd exp	1	−883.575	−0.88	0.383
1st exp × 2nd exp	1	1102.575	1.10	0.278
Sex × 1st exp × 2nd exp	1	1818.675	1.82	0.078

Discussion

In the sex-role reversed pipefish *Syngnathus typhle*, males and not females have the more active immune system. For the first time, we provide support for the notion that widespread immune dimorphism among the animal kingdom depends on life history and resource allocation patterns and not on sex per se, which follows from a life history trade-off view on Bateman's principle (Rolff, 2002). Our findings of reversed immune dimorphism apply to both correlative field data collected in four different populations and to a controlled experiment with a fully factorial primary and secondary exposure to a ubiquitous bacterium. We assessed several cellular and one humoral immune parameters (experiment only), which collectively support the initial hypothesis, namely that males display a higher immune activity than females (Table 5).

Absolute lymphocyte numbers, the lymphocyte index and the lymphocyte-to-monocyte ratio were higher for males than for females in both experiments. This suggests that males have more cells and a larger proportion of cells from the adaptive immune system than females. In mammals with conventional sex roles,

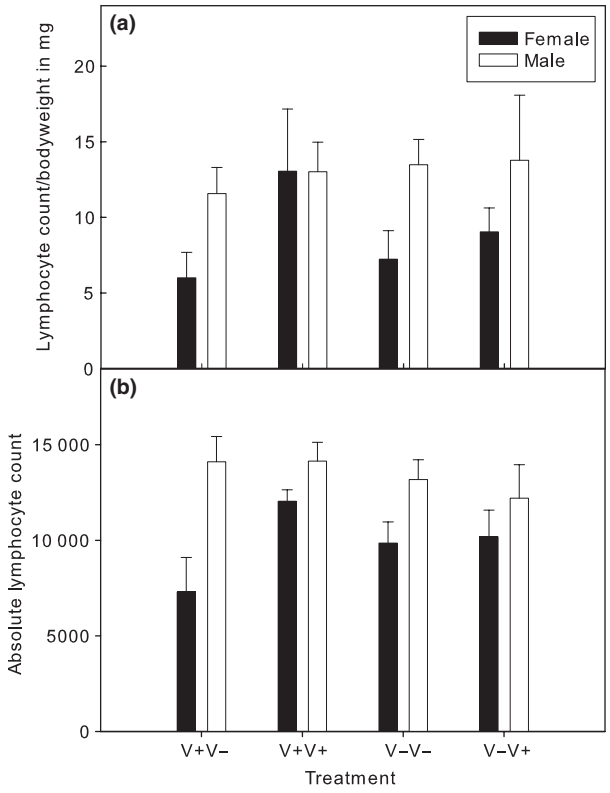

Fig. 2 Lymphocyte index (absolute lymphocyte count/bodyweight in mg) (a) and absolute lymphocyte count (b) for female (black) and male (light) pipefish fully subjected to heat-killed *Vibrio spp* in a factorial design (sham and no-sham) upon first and secondary exposure (treatment abbreviations: V+V−: first exposure *Vibrio*, secondary exposure sham injection; V+V+: both exposures *Vibrio*; V−V−: both exposures sham treatments; V−V+ first exposure sham injection, secondary exposure *Vibrio* injection). Bars show mean + SE.

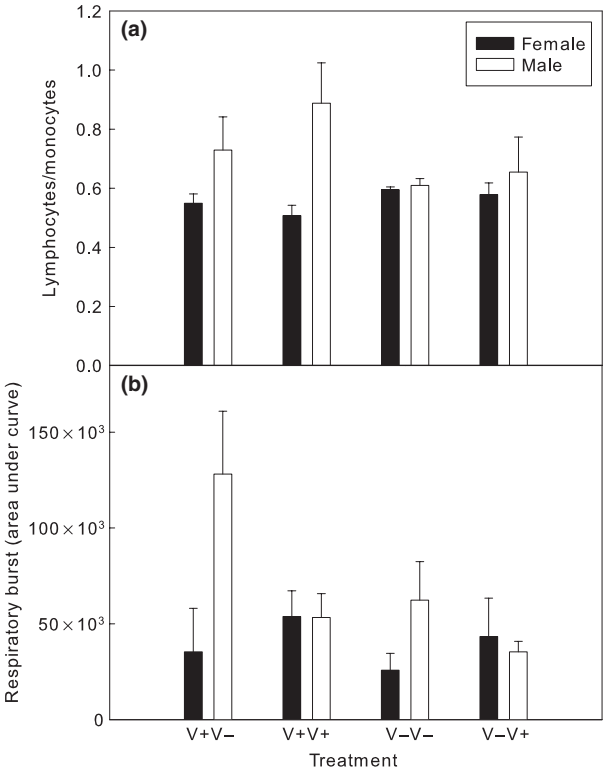

Fig. 3 Immune response measurements for female (black) and male (light) pipefish fully subjected to heat-killed *Vibrio spp* in a factorial design (sham and no-sham) upon first and secondary exposure (treatment abbreviations: V+V−: first exposure *Vibrio*, secondary exposure sham injection; V+V+: both exposures *Vibrio*; V−V−: both exposures sham treatments; V−V+ first exposure sham injection, secondary exposure *Vibrio* injection). Lymphocyte-to-monocyte ratio (a) is an indicator for the proportion of immune cells of the adaptive immune system (lymphocytes) to the proportion of immune cells of the innate immune system (monocytes). Respiratory burst (b) shows the phagocytic activity of monocytes. Bars show mean + SE.

lymphocyte counts were shown to be higher for females whereas neutrophil counts (cells of the innate immune system) were higher in males, suggesting the sex with a higher investment in reproduction to have more cells from the adaptive immune system (Nunn *et al.*, 2009).

Males not only had more proliferating lymphocytes but also a higher absolute lymphocyte count, demonstrated by the lower number of cells in the resting stage ($G_{0–1}$ phase) and a higher number of cells in the proliferating phases (S Phase and $G_{2–M}$ phase). That males had an induced activity of the adaptive immune system was found in the experimental data and partly (lower number of cells in the resting stage ($G_{0–1}$ phase)) also in the field data. Even though males possessed a lower proportion from cells of the innate immune system (monocytes) than females, they had a stronger respiratory burst when standardizing to absolute cell counts, suggesting a more efficient phagocytosis activity.

For the humoral immune response that was assessed here as antimicrobial activity of pipefish plasma measured directly against different strains of *Vibrio* spp., females inhibited growth of novel *Vibrio* stronger than males in two strains assessed. However, if antimicrobial activity was measured against the *Vibrio* strain that was used in the experiment for the fully reciprocal priming and challenge design, no sexual dimorphism could be found, suggesting that males catch up with the antimicrobial activity of females after a previous exposure. Furthermore, if the overall model is split and 2 × PBS-injected animals are compared, females showed higher (basal, noninduced) humoral activity than males to all three *Vibrio* strains used here. Conversely, if only animals are compared that were exposed to *Vibrio* twice, males showed a higher humoral activity than females against the *Vibrio* strain the animals were immunized with and

Table 2 Analysis of the effects of sex and *Vibrio* priming vs. sham injection (during 1st or 2nd exp) on immune parameters using three-way factorial ANOVA. Response variables are lymphocyte-to-monocyte ratio (a) and cell cycle (S phase) (b). *$P < 0.05$.

ANOVA				d.f.
Model				7
Error				32
Total				39
	d.f.	Estimate	*t* ratio	*P*
(a) Lymphocytes/Monocytes				
Sex	1	−0.096	−3.12	0.004*
1st exp	1	−0.045	−1.46	0.156
Sex × 1st exp	1	0.066	2.16	0.038*
2nd exp	1	−0.064	−2.08	0.046*
Sex × 2nd exp	1	−0.014	−0.44	0.663
1st exp × 2nd exp	1	0.001	0.02	0.985
Sex × 1st exp × 2nd exp	1	−0.063	−2.06	0.047*
(b) cell cycle S Phase				
Sex	1	−0.018	−2.69	0.011*
1st exp	1	0.011	1.54	0.134
Sex × 1st exp	1	0.005	0.77	0.445
2nd exp	1	−0.001	−0.21	0.832
Sex × 2nd exp	1	−0.001	−0.15	0.885
1st exp × 2nd exp	1	0.009	1.33	0.194
Sex × 1st exp × 2nd exp	1	0.006	0.80	0.427

Table 3 Analysis of the effects of sex and *Vibrio* priming vs. sham injection (during 1st or 2nd exp) on immune parameters using three-way factorial ANOVA. Response variable is respiratory burst. Interactions with $P > 0.60$ were removed from the analysis. *$P < 0.05$.

ANOVA				d.f.
Model				5
Error				33
Total				38
Effect tests	d.f.	Estimate	*t* ratio	*P*
Sex	1	−13844.7	−2.72	0.010*
1st exp	1	−77.87.2	−1.51	0.14
Sex × 1st exp	1	5562.3	1.08	0.287
2nd exp	1	2370.9	0.47	0.644
Sex × 2nd exp	1	−12354.3	−2.43	0.021*

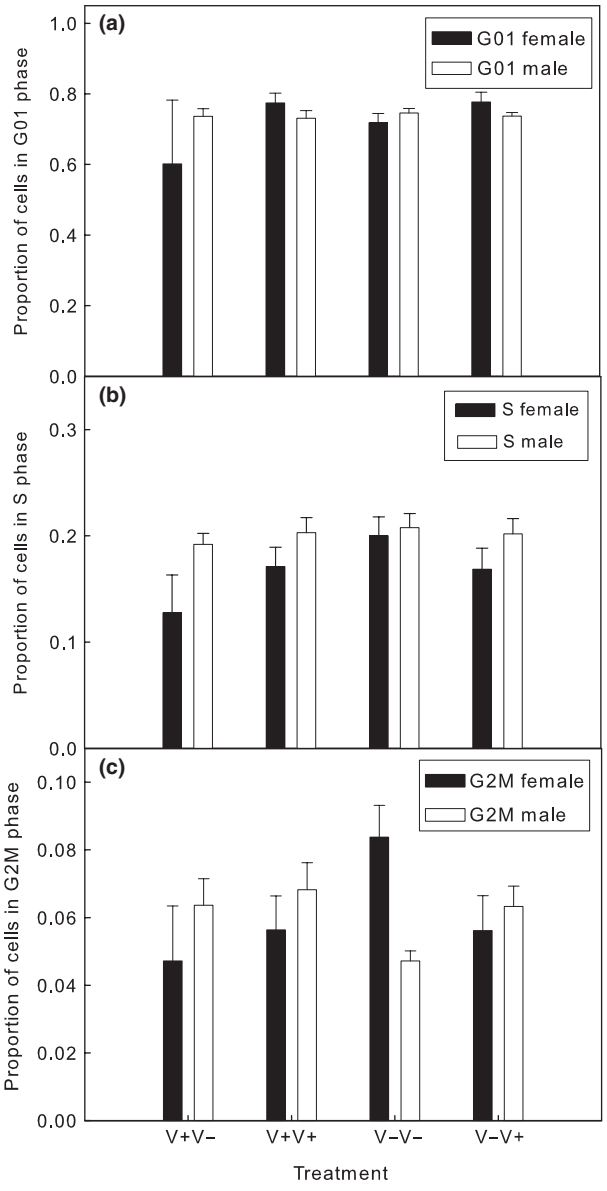

Fig. 4 Cell cycle analysis of head kidney leucocytes from female (black) and male (light) pipefish fully factorial injected with heat-killed *Vibrio spp* upon first and secondary exposure (V+V−: first exposure *Vibrio*, secondary exposure sham injection; V+V+: both exposures *Vibrio*; V−V−: both exposures sham treatments; V−V+ first exposure sham injection, secondary exposure *Vibrio* injection). G01 indicates resting phase of lymphocytes, and S and G2M indicate the proportion of proliferating lymphocytes. Bars show mean + SE.

also against the closely related other Italian *Vibrio* strain. Whereas intraperitoneal immunization with I11Ma2 affected females only weakly, males showed a clear increase in the lymphocyte-to-monocyte ratio. Males that were homologously exposed twice to an intraperitoneal injection with the same heat-killed *Vibrio* strain showed highest immune response (Fig. 2). This suggests that specificity and immune memory may occur upon secondary exposure to the same *Vibrio* strain in males but not in females, which was statistically supported by a significant three-way interaction.

Humoral activity against bacteria may depend on both innate immunity (e.g. lysozyme), which is more unspecific, and highly specific adaptive immunity (i.e. antibody-mediated complement activation). Here, the latter would be induced as a specific response to a previous challenge, whereas the first would rather be indicative of a general upregulation of innate humoral immunity,

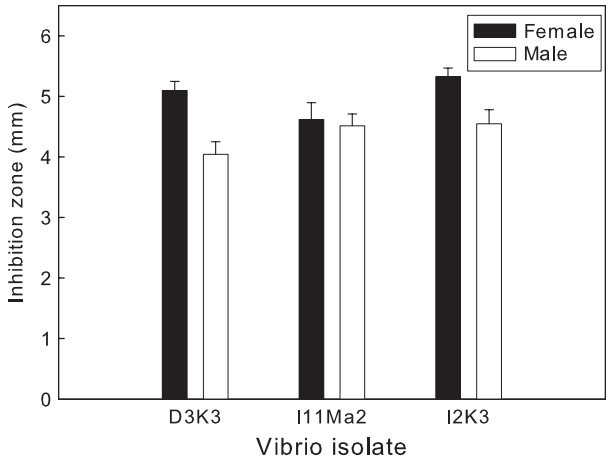

Fig. 5 Antimicrobial activity of female (black) and male (light) pipefish injected with heat-killed *Vibrio spp* (vs. sham injection) in a fully factorial design upon first and secondary exposure (V+V−: first exposure *Vibrio*, secondary exposure sham injection; V+V+: both exposures *Vibrio*; V−V−: both exposures sham treatments; V−V+ first exposure sham injection, secondary exposure *Vibrio* injection). The antimicrobial activity of pipefish plasma was tested against 3 *Vibrio* strains that were either novel (D3K3 and I2K3) or used in the experiment (I11Ma2). Bars show mean + SE.

Table 4 Analysis of the effects of sex and *Vibrio* priming vs. sham injection (during 1st or 2nd exp) on immune parameters using three-way factorial ANOVA. ANOVAS test for antimicrobial activity against the *Vibrio* strain used during the experiment (I11Ma2) and against two novel strains (D3K3 and I2K3). $*P < 0.05$.

ANOVA				d.f.
Model				7
Error				29
Total				36
Effect tests	d.f.	D3K3	I11Ma2	I2K3
Sex	1	< 0.001*	0.820	0.013*
1st exp	1	0.928	0.882	0.398
Sex × 1st exp	1	0.871	0.502	0.956
2nd exp	1	0.178	0.904	0.848
Sex × 2nd exp	1	0.824	0.780	0.036*
1st exp × 2nd exp	1	0.601	0.119	0.691
Sex × 1st exp × 2nd exp	1	0.766	0.061	0.601

Table 5 Overview of the immune parameters measured in this study. It is indicated whether males or females have a stronger (+) or weaker (−) or equal (=) immune response as the corresponding sex.

	Monocyte index	Respiratory burst	Cell Cycle (S Phase)	Lymphocyte	Antimicrobial activity
Males	=	+	+	+	−
Females	=	−	−	−	+

resulting in a broader (cross-reactive) immunocompetence. Our observation that females show more cross-reactivity supports a more important function of innate humoral immunity, whereas males use adaptive humoral immunity to clear the specific *Vibrio* strain they were challenged with. While the overall outcome might be identical (i.e. killing of the pathogen), an activation of the adaptive humoral immunity component might be more cost-effective compared to a broad immune stimulation.

The four study populations (Italy, Germany, Denmark and Sweden) can genetically only be differentiated into a Northern (Germany, Denmark and Sweden) and a Southern population (Italy) according to the polymorphism displayed at 10 neutral microsatellite loci. However, the differences in the immune parameters suggest that all four populations are exposed to different parasite infection pressures. In particular, Italian pipefish showed a higher lymphocyte-to-monocyte ratio than the Northern pipefish. Italian pipefish experience higher temperatures throughout the year, what may increase the parasite prevalence, which is also supported by the highest *Vibrio* spp prevalence (65%). The opposite was demonstrated for the most Northern pipefish population sampled, Sweden (Skagerrak area). Here, pipefish had a higher respiratory burst than fish of all other populations; their adaptive immune system on the other hand demonstrated a low activity.

This study explicitly focused on immune activation, using the injection of heat-killed bacteria to prevent confounding of results with possible virulence costs upon infection. Thus far, it is unknown whether or not parasite prevalence varies among the sexes in nature. In an analogous manner to species with conventional sex roles where males often reveal higher parasite prevalence, we would expect female pipefish to suffer from more parasites.

Our data indicate that in a sex-role reversed pipefish, males do not only have a more activated immunocompetence but also a more specific immune response upon a secondary exposure. Sexual immune dimorphism can thus be concluded to mainly depend on parental investment and not on sex per se, as the sex that maximizes fitness via longevity may indeed depend on a stronger immune response. However, to draw more general conclusions about Bateman's principle and immunity, studies on sex-role reversed animals should be expanded to cover the full degree of sex-role reversal. For this, Syngnathids are an ideal taxonomic group as they display a continuum from conventional sex roles to complete reversal.

Acknowledgments

We thank all the helping hands in the field and in fish dissection rounds: Susanne Landis, Chantal Kabus, Jens

Müller, Kristine Brüggemann, Philipp Schubert, August Hämmerli, Katrin Beining, Sören Bolte, Roman Asshoff and Niggi Weber. We also thank our technicians Rudi Lüthje and Svend Mees, who built excellent aquarium facilities for our fish. Thanks to Eva Phillip and the Inflammation Center at the University of Kiel for access to the flow cytometer facilities. We are grateful for useful comments by three anonymous reviewers that substantially improved the flow of this manuscript. OR was supported by a grant from the Volkswagen foundation programme 'Strengthening Evolutionary Biology'. In addition, this work was supported by a start-up grant from the Kiel excellence cluster 'The Future Ocean' to TBHR and OR.

References

Alcaide, E.D., Amaro, C. & Silveira, L. 2001. *Vibrio harveyi* causes disease in seahorse, *Hippocampus* sp. *J. Fish Dis.* **24**: 311–313.

Andersson, M. 1994. *Sexual Selection*. Princeton, New York.

Arnold, S.J. 1994. *Multivariate Inheritance and Evolution: A Review of Concepts*. University of Chicago Press, Chicago.

Austin, B. & Austin, D. 1999. *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*. Springer-Verlag, Berlin.

Balcazar, J., Gallo-Bueno, A., Planas, M. & Pintado, J. 2010a. Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from captive-bred seahorses with disease symptoms. *A van Leeuw J. Microbiol.* **97**: 201–210.

Balcazar, J., Lee, N.M., Pintado, J. & Planas, M. 2010b. Phylogenetic characterization and in situ detection of bacterial communities associated with seahorses (*Hippocampus guttulatus*) in captivity. *Syst. Appl. Microbiol.* **33**: 71–77.

Bateman, A.J. 1948. Intra-sexual selection in *Drosophila*. *Heredity* **2**: 349–368.

Berglund, A. & Rosenqvist, G. 1993. Selective males and ardent females in pipefish. *Behav. Ecol. Sociobiol.* **32**: 331–336.

Berglund, A., Rosenqvist, G. & Svensson, I. 1986a. Mate choice, fecundity and sexual dimorphism in two pipefish species (Syntnathidae). *Behav. Ecol. Sociobiol.* **19**: 301–307.

Berglund, A., Rosenqvist, G. & Svensson, I. 1986b. Reversed sex roles and parental energy investment in zygotes of two pipefish (Syngnathidae) species. *Mar. Ecol-Progr Ser.* **29**: 209–215.

Berglund, A., Rosenqvist, G. & Svensson, I. 1989. Reproductive success of females limited by males in two pipefish species. *Am. Nat.* **133**: 506–516.

Berglund, A., Widemo, M.S. & Rosenqvist, G. 2005. Sex-role reversal revisited: choosy females and ornamented, competitive males in a pipefish. *Behav. Ecol.* **16**: 649–655.

Berglund, A., Rosenqvist, G. & Robinson-Wolrath, S. 2006. Food or sex – males and females in a sex role reversed pipefish have different interests. *Behav. Ecol. Sociobiol.* **60**: 281–287.

Bilbo, S. & Nelson, R. 2001. Sex steroid hormones enhance immune function in male and female Silberian hamsters. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **280**: 207–213.

Braude, S., Tang-Martinez, Z. & Taylor, G. 1999. Stress, testosterone and the immunoredistribution hypothesis. *Behav. Ecol.* **10**: 345–350.

Caipang, C.M.A., Hynes, N., Puangkaew, J., Brinchmann, M.F. & Kiron, V. 2008. Intraperitoneal vaccination of Atlantic cod – expression of immune response genes. *Fish Shellfish Immunol.* **24**: 314–322.

Clutton-Brock, T.H. 1988. *Reproductive Success*. University of Chicago Press, Chicago.

Clutton-Brock, T.H. & Vincent, A.C.J. 1991. Sexual selection and the potential reproduction rates of males and females. *Nature* **351**: 58–60.

Folstad, I. & Karter, A. 1992. Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* **139**: 603–622.

Hamilton, W.D. & Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? *Science* **218**: 384–387.

Heidelberg, J., Heidelberg, K. & Colwell, R. 2002. Bacteria of the gamma-subclass Proteobacteria associated with zooplankton in Cheasapeake Bay. *Appl. Environ. Microbiol.* **68**: 5498–5507.

Hjeltnes, B. & Roberts, R. 1993. *Vibriosis*. Blackwell scientific, Oxford.

Iyer, P. & Roughgarden, J. 2008. Gametic conflict versus contact in the evolution of anisogamy. *Theor. Popul. Biol.* **73**: 461–472.

Jones, A.G. & Avise, J.C. 1997. Polygynandry in the dusky pipefish *Syngnathus floridae* revealed by microsatellite markers. *Evolution* **51**: 1611–1622.

Jones, A.G., Rosenqvist, G., Berglund, A., Arnold, S.J. & Avise, J.C. 2000. The Bateman gradient and the cause of sexual selection in a sex-role-reversed pipefish. *Proc. R. Soc. Lond. Ser. B* **267**: 677–680.

Jones, A.G., Rosenqvist, G., Berglund, A. & Avise, J.C. 2005. The measurement of sexual selection using Bateman's principles: an experimental test in the sex-role-reversed pipefish *Syngnathus typhle*. *Integr. Comp. Biol.* **45**: 874–884.

Klein, S.L. 2000. Hormones and mating system affect sex and species differences in immune function among vertebrates. *Behav. Process.* **51**: 149–166.

Kurtz, J. & Sauer, K. 2001. Gender differences in phenoloxidase activity of *Panorpa vulgaris* hemocytes. *J. Invertebr. Pathol.* **78**: 53–55.

Kurtz, J., Wiesner, A., Götz, P. & Sauer, K. 2000. Gender differences and individual variation in the immune system of the Scorpionfly *Panorpa vulgaris* (Insecta: Mecoptera). *Dev. Comp. Immunol.* **24**: 1–12.

Kurtz, J., Kalbe, M., Aeschlimann, P., Häberli, M., Wegner, K.M., Reusch, T.B.H. *et al.* 2004. Major histocompatibility complex diversity influences parasite resistance and innate immunity in sticklebacks. *Proc. R. Soc. Lond. Ser. B* **271**: 197–204.

Matsunaga, T. & Rahman, A. 1998. What brought the adaptive immune system to vertebrates? The jaw hypothesis and the seahorse *Immunol. Rev.* **166**: 177–186.

McKean, K.A. & Nunney, L. 2001. Increased sexual activity reduces male immune function in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* **98**: 7904–7909.

Nunn, C.L., Lindenfors, P., Pursall, E.R. & Rolff, J. 2009. On sexual dimorphism in immune function. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **364**: 61–69.

Parker, G., Baker, R. & Smith, V. 1972. The origin and evolution of gamete dimorphism and the male-female phenomenon. *J. Theor. Biol.* **36**: 529–553.

Peters, A. 2000. Testosterone treatment is immunosuppressive in superb fairy-wrens, yet free-living males with high testosterone are more immunocompetent. *Proc. R. Soc. Lond. Ser. B* **267**: 883–889.

Roberts, M., Buchanan, K. & Evans, M. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. *Anim. Behav.* **68**: 227–239.

Rolff, J. 2002. Bateman's principle and immunity. *Proc. R. Soc. Lond. Ser. B* **269**: 867–872.

Rosenberg, E. & Ben-Hain, Y. 2002. Microbial diseases of corals and global warming. *Environ. Microbiol.* **4**: 318–326.

Roth, O., Ebert, D., Vizoso, D.B., Bieger, A. & Lass, S. 2008. Male-biased sex-ratio distortion caused by *Octosporea bayeri*, a vertically and horizontally-transmitted parasite of *Daphnia magna*. *Int. J. Parasitol.* **38**: 969–979.

Sawabe, T., Setogushi, N., Inoue, S., Tanaka, R., Ootsubo, M., Yoshimizu, M. *et al.* 2003. Acetic acid production of *Vibrio halioticoli* from alingate: a possible role for establishment of albalone – *V. halioticoli* association. *Aquaculture* **54**: 843–846.

Scharsack, J.P., Kalbe, M., Derner, R., Kurtz, J. & Milinski, M. 2004. Modulation of granulocyte responses in three-spined sticklebacks (*Gasterosterus aculeatus* L.) infected with the tapeworm (*Schistocephalus solidus*, Muller 1776). *Dis. Aquat. Org.* **59**: 141–150.

Scharsack, J.P., Kalbe, M., Harrod, C. & Rauch, G. 2007a. Habitat specific adaptation of immune responses of stickleback (*Gasterosterus aculeatus*) lake and river ecotypes. *Proc. R. Soc. Lond. Ser. B* **274**: 1523–1532.

Scharsack, J.P., Koch, K. & Hammerschmidt, K. 2007b. Who is in control of the stickleback immune system: interactions between *Schistocephalus solidus* and its specific vertebrate host. *Proc. R. Soc. Lond. Ser. B* **274**: 3151–3158.

Schuurs, A.H.W.M. & Verheul, H.A.M. 1990. Effects of gender and sex steroids on the immune response. *J. Steroid. Biochem.* **35**: 157–172.

Scott, A.L. & Klesius, P.H. 1981. Chemiluminescence: a novel analysis of phagocytosis in fish. *Dev. Biol. Stand.* **49**: 243–254.

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

Sheridian, L.A.D., Poulin, R., Ward, D. & Zuk, M. 2000. Sex differences in parasitic infections among arthropod hosts: is there a male bias? *Oikos* **88**: 2523–2527.

Siva-Jothy, M.T. 2000. A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *Proc. R. Soc. Lond. Ser. B* **267**: 2523–2527.

Stearns, S.C. (Ed.) (1987) *Why Sexes Evolved and the Difference it Makes*. Birkhauser, Boston.

Tort, L.B.J. & Mackenzie, S. 2003. Fish immune system: a crossroads between immune and adaptive responses. *Immunologia* **22**: 277–286.

Trivers, R.L. 1972. *Parental Investment and Sexual Selection*. Aldine Press, Chicago.

Underwood, A.J. 1997. *Experiments in Ecology – Their Logical Design and Interpretation Using Analysis of Variance*. University Press, Cambridge.

Vandenberghe, J., Thompson, F., Gomez-Gil, B. & Swings, J. 2003. Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems. *Aquaculture* **219**: 9–20.

Wersto, R.P., Chrest, F.J., Leary, J.F., Morris, C., Stetler-Stevenson, M.A. & Gabrielson, E. 2001. Doublet discrimination in DNA cell-cycle analysis. *Cytometry* **46**: 296–306.

Williams, G.C. 1966. *Adaptation and Natural Selection*. Princeton University Press, Princeton.

Williams, G.C. 1975. *Sex and Evolution*. Princeton University Press, Princeton.

Wilson, A.B. & Veragut, I.E. 2010. The impact of Pleistocene glaciation across the range of a widespread European coastal species. *Mol. Ecol.* **19**: 4535–4553.

Zuk, M. & Mc Kean, K.A. 1996. Sex difference in parasite infections: patterns and processes. *Int. J. Parasitol.* **26**: 1009–1024.

Zuk, M. & Stoehr, A. 2002. Immune defense and host life history. *Am. Nat.* **160**: S9–S22.

Received 2 February 2011; revised 5 March 2011; accepted 14 March 2011