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REPORT

Density-dependent prophylaxis in the coral-eating crown-of-thorns sea star, *Acanthaster planci*

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Abstract The density-dependent prophylaxis hypothesis predicts that individuals at high density will invest more resources into immune defence than individuals at lower densities as a counter-measure to density-dependent pathogen transmission rates. Evidence has been found for this hypothesis in insects, but not in a non-arthropod taxon. To investigate this hypothesis in the coral-eating crown-of-thorns sea star, *Acanthaster planci*, density treatments were set up over 21 days, and pathogen infection was simulated with bacterial injection. Five immune responses: amoebocyte count, amoebocyte viability, lysosomal membrane integrity, respiratory burst and peroxidase activity were all upregulated at high density. These results demonstrate that immune investment shows phenotypic plasticity with adult population density in agreement with the density-dependent prophylaxis hypothesis. Here I show that the density-dependent prophylaxis hypothesis is neither dependent on larval density nor restricted to insects, and hence may potentially have important consequences on disease dynamics in any species with widely fluctuating population densities. This is the first demonstration of the density-dependent prophylaxis hypothesis outside arthropods.

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Introduction

Parasites and pathogens pose a threat to all animals and plants and thus represent an important selective force on the immune response of their hosts (Wilson and Cotter 2008). Parasites are implicated in multiple evolutionary processes including the evolution of host optimal life-history strategies (e.g., Sheldon and Verhulst 1996). Disease may be a density-dependent factor (Alexander 1974; Møller et al. 1993; Moore 2002) due to the greater contact among conspecifics at high densities (Steinhaus 1958) and increased horizontal transmission of viral, bacterial, protozoan and fungal infections (Anderson and May 1979, 1981; McCallum et al. 2001). Evidence for density-dependent disease risk is widespread in mammals (Freeland 1979; Hoogland 1979), birds (Brown and Brown 1986; Shields and Crook 1987), insects (Dwyer and Elkinton 1993; Knell et al. 1996; Lindsey et al. 2009), echinoderms (Lessios 1988; Lafferty 2004) and molluscs (Lafferty and Kuris 1993). As such, organisms that experience high population densities or wide fluctuations in population density would benefit from higher disease resistance; however, immunity is costly to maintain and express (Sheldon and Verhulst 1996; Kraaijeveld and Godfray 1997).

As a counter measure to density-dependent pathogen transmission rates and costly disease resistance (e.g., Lochmiller and Deerenberg 2000), natural selection should favor phenotypically plastic prophylactic investment in disease resistance mechanisms, a phenomenon known as density-dependent prophylaxis (DDP) (Reeson et al. 1998;

Wilson and Reeson 1998; Wilson et al. 2002). Insects have been found to use early larval density to predict the risk of exposure to pathogens and optimally allocate resources to pathogen resistance in either adults or later larval instars (Wilson and Reeson 1998). Insects show widespread evidence for DDP from examination of their susceptibility to viruses (Mitsui and Kunimi 1988; Kunimi and Yamada 1990; Reeson et al. 2000), their greater investment in immune function (Reeson et al. 1998; Barnes and Siva-Jothy 2000; Wilson et al. 2001, 2002; Cotter et al. 2004) and increased cuticular melanization (Reeson et al. 1998; Barnes and Siva-Jothy 2000). At low population densities, individuals should reallocate these resources to other functions, such as growth or reproduction (Kraaijeveld and Godfray 1997; Webster and Woolhouse 1999; Rolff and Siva-Jothy 2003). The DDP hypothesis assumes that all traits associated with disease resistance should be up-regulated in response to an increased threat of disease at high density; however, trade-offs within the immune system have been found to exist (Cotter et al. 2004). Recently, DDP is considered to have a much broader significance independent of larval conditions, as shown by the labile immune responses to rapidly changing conditions in adult social insect populations (Ruiz-Gonzalez et al. 2009). In contrast to DDP, high density conditions may increase intra-specific competition and induce physiological or nutritional stress, increasing host susceptibility to infection and referred to as the crowding-stress hypothesis (Steinhaus 1958; Lindsey et al. 2009).

To date, the phenomenon of DDP has not been examined in a non-arthropod taxon. Echinoderms have important functions in marine ecosystems, and 11 species show marked fluctuations in population density from very high to very low densities (Uthicke et al. 2009). Such population density fluctuations are sometimes cyclic, often on a decadal scale, for example, for the coral-eating crown-of-thorns sea star, *Acanthaster planci*, a keystone species that occurs in the tropical Indo-Pacific from Panama to the Red Sea (Uthicke et al. 2009). *Acanthaster planci* exhibits marked repeated pulses of increased population density called "outbreaks"—the sudden emergence of a large population after a period of relative rarity (Moran 1986). *Acanthaster planci* generally occurs in low densities of ~0.06 individuals/ha (Endean 1974); however, up to 53,750 individuals per km^2 have been recorded during an outbreak in French Polynesia (Kayal et al. 2011) and such outbreaks have caused declines in coral reefs worldwide: Great Barrier Reef (Pratchett 2005), Japan (Yamaguchi 1986), Hawaii (Branham et al. 1971) and Micronesia (Colgan 1987). Echinoderms that are broadcast spawners with planktotrophic larvae show a higher propensity for large population density variations (Uthicke et al. 2009) and the removal of larval food limitation causes primary population outbreaks of *A. planci* on the Great Barrier Reef (Fabricius et al. 2010). Outbreaks on individual reefs usually end when most palatable corals are consumed (Uthicke et al. 2009).

Dense populations of another echinoderm, the sea urchin, are more likely to experience epidemics and suffer higher mortality (Lafferty 2004); therefore, we assume that *A. planci* would also exhibit density-dependent disease risk. We predict that *A. planci* should, in theory, benefit from tailoring immunity levels with population density. Disease resistance has not been measured in *A. planci*; therefore, immune measures were optimized and the DDP hypothesis tested. One specific prediction of the DDP hypothesis is that as the degree of crowding increases, the per capita investment in disease resistance mechanisms will increase (Wilson and Reeson 1998). In this paper *A. planci* was kept at low and high densities with unlimited food, and their induced investment to immunity was measured following bacterial challenge.

As in insects, echinoderm immunity consists of both cellular and humoral responses, but whereas echinoderm cellular responses are mediated by coelomocytes, free roaming cells in the coelomic fluid, insect responses are mediated by hemocytes in the hemolymph (Boman and Hultmark 1987; Gillespie et al. 1997; Vilmos and Kurucz 1998; Ramírez-Gómez and García-Arrarás 2010; Smith et al. 2010). The overall response to infection or injury by insects involves hemocytes removing foreign material from circulation by cellular responses such as phagocytosis, encapsulation and nodule formation (Boman and Hultmark 1987). Humoral responses then trigger melanization (via the prophenoloxidase activating cascade), clotting of the hemolymph, production of reactive oxygen species and agglutination of invading microorganisms by lectins (Boman and Hultmark 1987; Gillespie et al. 1997). RNA and specific antibacterial peptides and proteins are synthesized and secreted into the hemolymph (e.g., cecropins, attacins, lysozyme, defensins and diptericins) killing foreign cells by lysis and halting the infection process (Boman and Hultmark 1987; Vilmos and Kurucz 1998). Echinoderm immunity shows similar responses but mediated by different cells and molecules. Echinoderm coelomocytes secrete cellular products for pathogen phagocytosis and encapsulation (e.g., phagocytes, spherule cells and vibratile cells) and agglutination factors that mediate clotting (Smith et al. 2010). Humoral responses recognize and neutralize foreign material, promote cell migration and agglutination, play a role in wound healing and cause opsonization, oxidative defense and respiratory burst, for example, lectins, hemolysin, agglutinans (Smith et al. 2010; Ramírez-Gómez and García-Arrarás 2010). This paper tests whether high population density induces *A. planci* to invest more into five disease resistance mechanisms in support of the DDP hypothesis shown in insects.

Materials and methods

Study site

This study was conducted on Moorea, French Polynesia, a high island surrounded by a barrier reef system. A total of 150 *A. planci* were collected from the inner reef (reef flat) on the northern shore (17°28′438″S, 149°47′008″W) from a depth of 1–2 m. An unprecedented population outbreak of *A. planci* was documented at Moorea from 2006 to 2009 reaching a maximum of 53,750 individuals per km^2 on the outer reef (fore reef) in June 2008 (Kayal et al. 2011) and causing a 77% decrease in live coral cover (Leray et al. pers. comm.). *Acanthaster planci* were still evident on the inner reef (reef flat) in 2009–2010 with a maximum of 20 individuals per 200 m^2.

Bacterial challenge and peak response

To simulate bacterial invasion for immunity measures, 1 ml of a suspension of the dead Gram-positive bacteria *Micrococcus lysodeikticus* (10 mg/ml, Sigma M0508) in 0.1 M $NaHCO_3$ buffer, pH 9.0, was injected into each *A. planci* (approx. 0.06 ml per arm) (Bekri and Pelletier 2004).

In order to determine the timing of a peak response by *A. planci* to a bacterial challenge, amoebocyte concentration (mm^3; see below for methods) was measured daily over nine days following bacterial challenge (mean diameter ± SE = 13.4 ± 2.4 cm, $n = 5$) and saline injection as a control (mean diameter ± SE = 11.5 ± 1.3 cm, $n = 7$; no difference in mean diameter between groups; independent samples t test: $t_{1,10} = 0.787$, $p = 0.450$) (see Electronic Supplemental Material, ESM Appendix 1).

Density experiment

Experimental blocks consisted of a 2-m diameter outdoor tank (1,250 l) with constant running seawater. *Acanthaster planci* were randomly assigned to either the low density (1 individual per tank) or the high density (8 individuals per tank) treatment. *Acanthaster planci* spent 15 days at their treatment density prior to bacterial injection. Coelomic fluid was collected 6 days post-bacterial challenge corresponding to their peak immune response (ESM Appendix 1). Therefore, *A. planci* spent a total of 21 days at the treatment densities. *Acropora* spp and *Pocillopora cf. verrucosa* colonies of approx. 200 cm maximum diameter were provided ad libitum as food. Body diameter and number of arms were measured for each individual. There was no difference in body size between density treatments (GLMM: $F_{1,126} = 0.200$, $p = 0.656$; mean body diameter (±SE): low density = 14.9 ± 3.6 cm, $n = 23$; high density = 15.1 ± 2.2 cm, $n = 105$). A positive correlation was found between body diameter and number of arms of *A. planci* (Pearson correlation: $r = 0.48$, $N = 126$, $p < 0.001$).

Five trials were carried out over a period of 1 month (low/high density treatments per trial as follows = 3/3, 5/3, 5/3, 5/3 and 5/2) resulting in a total of 23 and 14 replicates of the low and high density treatments respectively. Mortality of seven individuals (which were replaced with new, but unchallenged individuals) from one high density treatment in trial 5 meant only 105 individuals were tested at high density. Therefore, 128 individuals were tested in total. Due to a temporary spectrophotometer malfunction during the first trial, measures of lysosomal membrane integrity, oxygen metabolism and peroxidase activity could not to be measured (3 low and 3 high density treatments).

Immunity measures

Solutions

Calcium- and magnesium-free artificial seawater (CMF ASW) was made up by adding 28.333 g NaCl, 0.665 g KCl, 3.916 g Na_2SO_4 and 0.194 g $NaHCO_3$ in 1l Nanopure water. Anti-aggregative solution (AG) was made up of CMFASW containing 15 mM ethylenediamine tetracetic acid (EDTA).

Coelomic fluid (CF) collection

Six days after bacterial challenge, *A. planci* were blotted with a sorbing paper and 1.5 ml of CF was withdrawn into an equal volume of cold AG by inserting a 3 ml pre-cooled syringe fitted with a sterile 23 gauge needle into the distal third of one arm. The resulting CF-AG suspension was homogenized immediately to prevent coagulation, thrombus formation and secretory degranulation of coelomocytes. The CF-AG suspension was then subdivided into five sub-samples in order to measure five aspects of immune function described below.

Amoebocyte count

Counts of amoebocytes were assessed using an Improved Neubauer© Bright-Line hemacytometer (Sigma Z359629, Analytic Lab, St Mathieu de Treviers) under a binocular microscope at magnification of 115x. The total number of amoebocytes in the middle square (0.1 mm × 1 mm × 1 mm) of both chambers of the hemacytometer was determined, and the mean of the two counts was recorded. The results took into account the dilution factor of the CF in the solution and are expressed as amoebocyte count per mm^3 (Coteur et al. 2003; Bekri and Pelletier 2004).

Samples from all 5 trials were measured resulting in 23 and 105 individuals, 23 and 14 replicates, for the low and high density treatments respectively.

Amoebocyte viability

The viability of amoebocytes was determined using the Trypan blue dye exclusion test. Trypan blue is a stain that only enters dead cells. 0.1 ml CF-AG suspension was added to an equal volume of a fresh solution of 0.2% Trypan blue (Sigma T8154, Analytic Lab, St Mathieu de Treviers) in AG solution. The percentage of viable cells was assessed in duplicate using an Improved Neubauer© Bright-Line hemacytometer under a binocular microscope at magnification of 115x (Bekri and Pelletier 2004). By measuring the cell numbers of both unlysed and lysed cells, we could determine the percentage of dead cells. Due to time constraints, only samples from trials 2, 3 and 4 were measured resulting in 14 and 63 individuals (one sample was lost), 14 and 8 replicates, for the low and high density treatments respectively.

Lysosomal membrane integrity (neutral red retention)

The neutral red (NR) retention assay is a measure of an individual's response to stress via the retention of a neutral red dye in lysosomes (Song et al. 2007). In unstressed cells, lysosomes will accumulate and retain neutral red dye for an extended period of time; however, once destabilized by a stress response, the neutral red dye will leak into the cytosol of the cell through the damaged lysosomal membrane (Lowe et al. 1995a, b). Lysosomal integrity of amoebocytes was evaluated using the method of the cationic probe NR retention as adapted from Bekri and Pelletier (2004). 0.1 ml of NR (Acros Organics 415490250) was added to 1 ml of CF-AG suspension. The samples were incubated in the dark at 18°C for 75 min. After centrifugation at 552×*g* for 5 min, the supernatant was removed and fixed in 400 µl of 5% formaldehyde in CMFASW during 15 min with mild manual shaking. After centrifugation, the fixative solution was removed and replaced by 1 ml CMFASW, and the preparation was stored in the dark at 4°C. Prior to the reading day, the supernatant was replaced by 1 ml of the extraction solution containing 1% (v/v) acetic acid + 50% (v/v) ethanol in Nanopure water, and cells were incubated for 15 min at room temperature with occasional shaking to release neutral red retained by lysosomes. The absorbance of neutral red extract was measured at 490 nm on a BioTek Microplate Reader ELx800UV (BioTek Instruments GmbH, Bad Friedrichshall, Germany) calibrated with the extraction solution. Results are expressed as units of optical density (OD) per well as well as OD per well per 1 million coelomocytes per mm^3. Due to a temporary spectrophotometer malfunction during the first trial, only 20 and 82 individuals, 20 and 11 replicates, for the low and high density treatments, respectively, were tested.

Oxygen metabolism of coelomocytes

This test determines whether immune system cells can change the colorless chemical nitroblue tetrazolium (NBT) into a deep blue color. 1.0 ml of CF-AG suspension was softly centrifuged at 100*g* for 10 min. The supernatant was removed and 400 µl of CMFASW added during 15 min with mild manual shaking. 100 µl of the cell suspension was placed into a 96-well flat-bottom microtiter plate (Fisher Scientific, Strasbourg) in duplicate. To determine the induced index of the NBT-test, 50 µl of 0.05% suspension of Zymosan A from *Saccharomyces cerevisiae* (Sigma, Z4250, Analytic Lab, St Mathieu de Treviers) in CMFASW was added. 50 µl of 0.2% *p*-NBT (Sigma, 84010, Analytic Lab, St Mathieu de Treviers) was added into all wells. The plates were incubated for 24 h at 26°C (local seawater temperature). The supernatant was removed, thereby terminating the reaction. To fix the samples, 50 µl of 95% ethanol (Fisher Scientific, M0346X) was added into each well. The plates were placed in a Diemos Fisherbrand thermostat at 37°C for 6–8 h until complete drying. The plates were stored at −20°C prior to spectrophotometric recording. On the reading day, the plates were defrosted, and 200 µl of distilled water was added into each well, and the plates were centrifuged at 400*g* for 7 min. The supernatant was removed, and 120 µl of 2 M KOH (Sigma, P5958, Analytic Lab, St Mathieu de Treviers) in water and 140 µl of dimethylsulfoxide (Sigma D8418, Analytic Lab, St Mathieu de Treviers) were added into each well. Complete dissolution of NBT diformazan granules was achieved by incubation of the plate on a shaker at 37°C for 1 h. The plates were read at 630 nm on a BioTek Microplate Reader ELx800UV (BioTek Instruments GmbH, Bad Friedrichshall, Germany). Results were expressed as units of optical density (OD) per well. Due to a temporary spectrophotometer malfunction during the first trial only 20 and 82 individuals, 20 and 11 replicates, for the low and high density treatments, respectively, were tested.

Peroxidase activity

50 µl of CF-AG suspension was added into wells of a 96-well flat-bottom microtiter plate (Fisher Scientific, Strasbourg) in duplicate. As a control, 50 µl of CMFASW was added. 50 µl of the chromogenic mixture containing 0.4% orthophenylenediamine dihydrochloride (OPD) (Sigmafast™ OPD, P9187, Analytic Lab, St Mathieu de

Treviers) was added into each well. Sigmafast™ OPD tablets have the following concentrations in solution: 0.4 mg/ml OPD, 0.05 M phosphate-citrate, pH 5.0 and 0.4 mg/ml urea hydrogen peroxide. The samples were incubated in darkness in a Diemos Fisherbrand thermostat at 37°C for 75 min. The reaction was terminated by the addition of 50 µl of 2 M sulfuric acid. Intensity of the reaction was determined at 490 nm using a BioTek Microplate Reader ELx800UV (BioTek Instruments GmbH, Bad Friedrichshall, Germany). Results were expressed as units of optical density (OD) per well. Due to a temporary spectrophotometer malfunction during the first trial only 20 and 82 individuals, 20 and 11 replicates, for the low and high density treatments, respectively, were tested.

Statistics

All immune responses showed normal distributions. Generalized linear mixed models were used to analyze the dependent variables, measures of immunity in coelomic fluid collected from *A. planci* six days post-bacterial challenge with low and high density treatments as a fixed effect. Random effects were trial (5 trials over 1 month) and experimental tank.

Results

Amoebocyte count

Repeatability was calculated for amoebocyte count prior to the density experiment. Repeatability for duplicate amoebocyte counts (mean of two counts measured twice from each CF sample; $n = 82$ individuals) using analyses of variance (Lessells and Boag 1987); repeatability (F ratio) = 0.883 (16.21). Repeatability for amoebocyte counts measured from the same individual over 2–8 consecutive days ($n = 14$ individuals) using analyses of variance; repeatability (F ratio) = 0.677 (9.52).

Immunity measures

The only covariation between immunity traits was found between the number of amoebocytes and lysosomal membrane integrity (Table 1).

Acanthaster planci held at high densities for 21 days produced significantly more circulating amoebocytes (Table 2a, Fig. 1a) of which a great percentage was alive (Table 2b, Fig. 1b) than those held at low density. Due to the significant positive correlation between the number of amoebocytes and lysosomal membrane integrity, NR was

Table 1 Covariance matrix (Pearson correlations) between immune responses measured in *Acanthaster planci* following bacterial challenge at low and high density

	Amoebocyte count	Amoebocyte viability	Lysosomal membrane integrity	Respiratory burst	Peroxidase activity
Amoebocyte count		0.193	0.269**	0.018	0.070
Amoebocyte viability	0.193		0.177	0.160	0.134
Lysosomal membrane integrity	0.269**	0.177		0.011	0.141
Respiratory burst	0.180	0.160	0.110		0.049
Peroxidase activity	0.070	0.134	0.141	0.049	

$N = 102$ for all cells except those correlated with amoebocyte viability where $N = 77$

** Refers to $p = 0.006$

Table 2 Generalized linear mixed model was used to analyze (a) amoebocyte concentration (mm^3) (b) amoebocyte viability (% live cells—trypan blue test) (c) lysosomal membrane integrity (neutral red retention—optical density) divided by amoebocyte concentration, (d) respiratory burst (optical density) and (e) peroxidase activity (optical density) of coelomic fluid collected from *Acanthaster planci* 6 days post-bacterial challenge and after 21 days in low or high density treatments

Source	(a) Amoebocyte count			(b) Amoebocyte viability			(c) Lysosomal membrane integrity			(d) Respiratory burst			(e) Peroxidase activity		
	*df*n, d	*F*	*p*	*df*n, d	*F*	*p*	*df*n, d	*F*	*p*	*df*n, d	*F*	*p*	*df*n, d	*F*	*p*
Density	**1,115**	**5.246**	**0.024**	**1,49**	**6.241**	**0.016**	**1,91**	**7.295**	**0.009**	**1,91**	**4.103**	**0.046**	**1,97**	**6.841**	**0.010**

Estimates and residuals for trial and experimental tank were as follows: (a) trial: 3.06×10^6 and 2.33×10^6, tank: 2.17×10^5 and 2.99×10^5, $N = 128$; (b) trial: 88.19 and 95.79, tank: 12.21 and 18.32, $N = 77$; (c) trial: 7.05×10^{10} and 1.6×10^{10}; and for tank: 3.3×10^{10} and 0.00, $N = 102$; (d) trial: 0.752 and 0.617, tank: 0.0 and 0.0, $N = 102$; (e) trial: 0.027 and 0.022, tank: 0.0 and 0.0, $N = 102$

df degrees of freedom for numerator (n) and denominator (d), F test statistic: p probability, significant values are highlighted in bold text

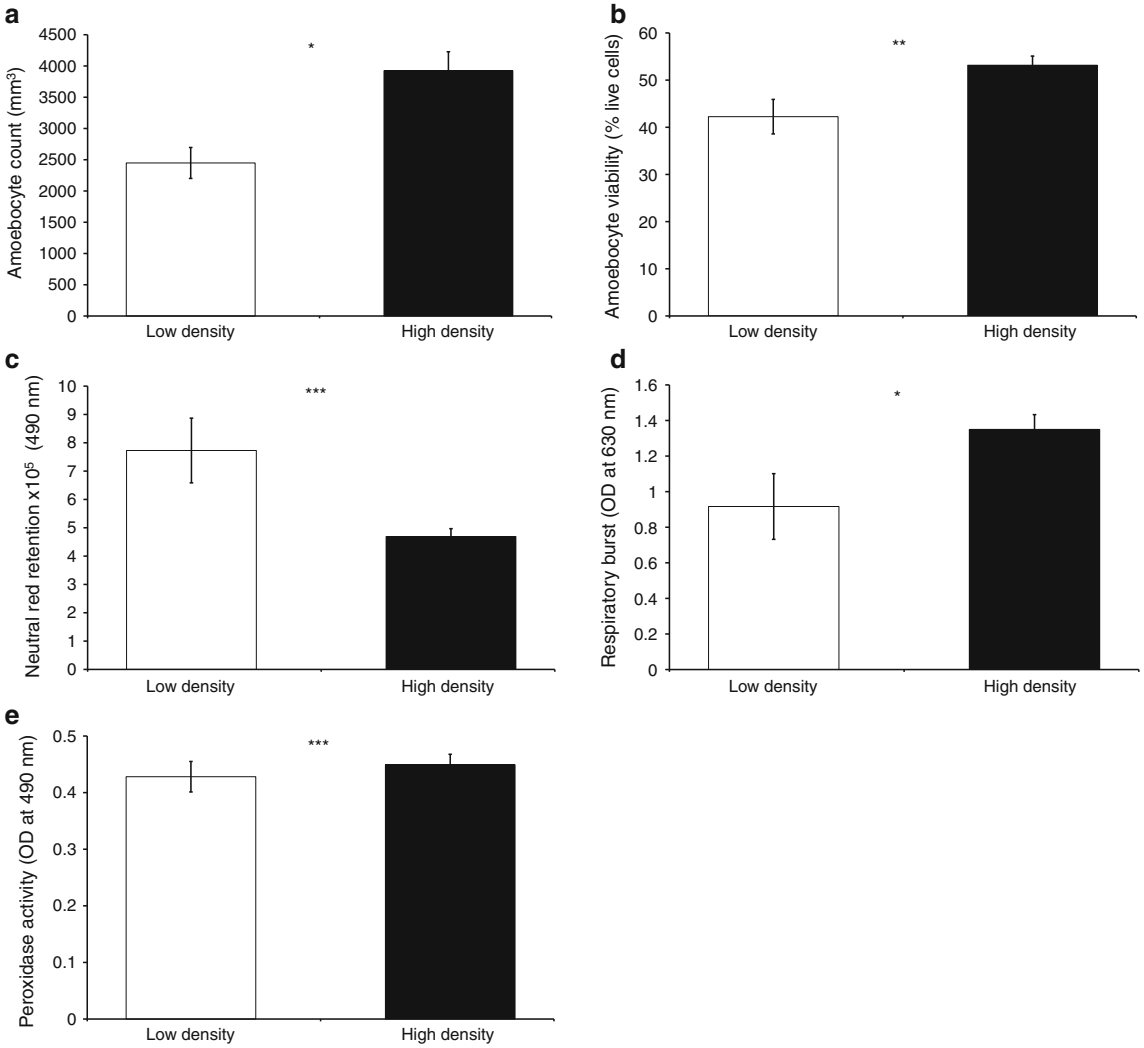

Fig. 1 Mean (±1 SE) **a** amoebocyte concentration (mm^3), **b** amoebocyte viability (% live cells—trypan blue test), **c** Neutral red retention (standardized optical density), **d** oxygen metabolism (standardized optical density) and **e** peroxidase activity (standardized optical density) of coelomic fluid collected from *Acanthaster planci* 6 days post-bacterial challenge and after 21 days in low (1 individual per tank) or high density (8 individuals per tank) treatments. Sample sizes are **a** 23 and 105, **b** 14 and 63, **c**, **d** and **e** 20 and 82 for low and high density respectively. $***p < 0.01$, $**p < 0.02$ and $*p < 0.05$. *Open square* low density and *filled square* high density

divided by number of amoebocytes. A significant effect of density on lysosomal membrane integrity was found (Table 2c, Fig. 1c), whereby NR was higher in *A. planci* from the low density than high density treatment. *Acanthaster planci* held at high densities for 21 days produced significantly more superoxide anions (O_2^-) (Table 2d, Fig. 1d) and had a greater peroxidase activity (Table 2e, Fig. 1e) than those held at low density.

Discussion

Density-dependent prophylaxis (DDP), in which individuals upregulate their parasite resistance mechanisms under conditions of conspecific crowding to counteract the increased risk of parasitic infection at high densities (Wilson and Cotter 2008), was examined in a population of the coral-eating crown-of-thorns sea star, *A. planci*, at Moorea, French Polynesia, which experiences frequent population outbreaks. Individuals at high density revealed both higher numbers of circulating amoebocytes and a higher percentage of live amoebocytes compared to those at low density. Lysosomal membrane integrity was higher at high density, and respiratory burst and peroxidase activity were also upregulated under high density. This is the first study to have found evidence for density-dependent prophylaxis in a non-arthropod taxon.

In echinoderms, cellular immune responses are carried out by circulating cells, coelomocytes, in coelomic cavities. Among these cells, the amoebocytes are the most

active in the reaction against non-self-material (Chia and Xing 1996), in eliminating pathogens, for example, sea-urchins (Yui and Bayne 1983), and have been well characterized in previous studies (Vanden Bossche and Jangoux 1976; Coteur et al. 2002). Amoebocytes participate in functions similar to their immune system homologs in vertebrates, such as the formation of cellular clots, phagocytosis of non-self-material and its subsequent destruction (Coteur et al. 2002), encapsulation (Jans et al. 1996), clearance of bacteria and other foreign materials, the production of reactive oxygen species (ROS) (Gross et al. 1999; Coteur et al. 2002), as well as oxygen transport. Not only did *A. planci* upregulate the number of circulating amoebocytes at high density, but also the percentage of live cells (Table 2a, b, Fig. 1a, b). Therefore, *A. planci* increases its main line of cellular defence against invading pathogens at high density.

The immune response of echinoderms is also involved in the mobilization of lysosomes that sequester and metabolize natural toxins (Bekri and Pelletier 2004). Lysosomes respond to both internal and external stress (Harding et al. 2004a, b) by destabilization, damage to their membrane, resulting in their contents leaking into the cytosol of the cell (Lowe et al. 1995a, b). Lysosomal integrity assays are a useful method of evaluating responses to environmental, physiological and mechanical stresses in oysters, mussels, freshwater snails, abalone and sea stars (Bekri and Pelletier 2004). The amount of neutral red was higher in *A. planci* at low density (Fig. 1c) demonstrating greater leakage through a damaged lysosomal membrane, suggesting that lysosomal integrity was stronger in *A. planci* at high density.

Amoebocytes engulf pathogens and trigger a cytotoxic mechanism generating a series of antimicrobial substances, called reactive oxygen species (ROS) (Chiu et al. 2007). This occurs by the activation of a membrane-bound enzyme complex, NADPH oxidase, which, after binding the cell to a foreign particle, reduces molecular oxygen into the highly reactive superoxide anion O_2^- (Babior 1984; Noel et al. 1993). This free radical can yield several highly toxic oxidants such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and numerous other reactive compounds (Babior 1984; Munoz et al. 2000). In response to stimulation by zymosan, the amoebocytes of *A. planci* increased superoxide anion (O_2^-) production at a greater rate at high density than at low density (Fig. 1d). An increase in superoxide anion production is considered to be beneficial with respect to enhancement of immunity (Munoz et al. 2000) and an increase in disease resistance (Song and Hsieh 1994), therefore, the results suggest that *A. planci* upregulated its respiratory burst in response to high density.

In the course of a defense reaction, coelomocytes also release numerous enzymes including peroxidase, which destroy invading microorganisms. Peroxidase activity modifies low density lipoproteins in the presence of H_2O_2 (Halliwell and Gutteridge 1998; Papas 1999) and has been detected in many groups of invertebrates including sea cucumbers (Canicatti 1990) and sea stars (Kudryavtsev et al. 2005). *Acanthaster planci* showed higher peroxidase activity at high density (Fig. 1e), further confirming the upregulation of the immune system in response to high density.

The immune system in a population of the crown-of-thorns sea star, *A. planci*, from Moorea, French Polynesia has been shown to be upregulated under conditions of high density. However, other endogenous factors such as age, gender and reproductive status may also condition the reaction of immune cells to a particular stimulus (Oliver and Fisher 1999; Kurtz et al. 2000). The sea stars used in the experiment were not sexed, nor their reproductive status assessed, therefore differences in gender and reproductive status between density treatments cannot be ruled out, although individuals were randomly assigned to treatments and the experiment was carried out over one month minimizing any seasonal reproductive effects, and no difference in size was observed between density treatments. Animals living at high density might also experience more intense competition for resources, and both physiological and nutritional stress have been proposed to make animals more susceptible to infectious diseases, the crowding-stress hypothesis (Steinhaus 1958; Lindsey et al. 2009). All the sea stars in this experiment were fed ad libitum throughout the experiment; therefore, physiological stress is the only likely factor affecting their immune response.

These results demonstrate that the crown-of-thorns sea star, *A. planci*, shows phenotypic plasticity in immunity in response to changing population density in support of the density-dependent prophylaxis (DDP) hypothesis (Wilson and Reeson 1998; Wilson et al. 2002). Phenotypic plasticity is the differential phenotypic expression of a genotype as a response to environmental cues (West-Eberhard 1989, 2003). In heterogeneous environments, it is unlikely that a single phenotype is able to achieve highest fitness under all environmental conditions, therefore plasticity is likely to be advantageous and favored by selection (Via et al. 1995). The immune response of multiple taxa (e.g., mammals, fish, insects) may be modulated by environmental cues such as temperature, photoperiod and resource limitation, enabling individuals to adjust to environmental stressors (Bowden et al. 2007; Moreno-Garcia et al. 2010). *Acanthaster planci* upregulate their immune response to increase disease resistance, however, whether this

upregulation results in increased survival, a specific prediction of DDP, is not known; however, previous evidence for DDP has also been based on investment in immune function (Reeson et al. 1998; Barnes and Siva-Jothy 2000; Wilson et al. 2001, 2002; Cotter et al. 2004). Central to life-history theory is the assumption that trade-offs frequently exist between the benefits afforded by plasticity in one trait and the consequences of that plasticity on other traits that affect fitness (Stearns 1976, 1989a, b, 1992; Newman 1992). Contrary to the trade-offs found within the immune system in Lepidopteran larvae (Cotter et al. 2004), the absence of any negative correlations between immune responses in this study (Table 1) suggests that no trade-offs are present in the sea star immune system for the responses measured. However, the upregulation of immunity may also indirectly lower other fitness traits to which it is causally linked (e.g., reproduction, Mills et al. 2009, 2010; Schroderus et al. 2010). We did not measure any other life-history traits in this study, but it would be interesting to monitor reproduction in *A. planci* in the future.

How does *A. planci* detect changes in population density? Individual desert locusts regularly touching others on the hind legs within populations that have become concentrated by the environment is the primary cause of switching from the solitarious to the gregarious phase (Simpson et al. 2001). Similarly, physical stimuli by other *A. planci* may cause individuals to change their behavior and upregulate their immune response. Alternatively, *A. planci* may use its neuroendocrine system to collect and process environmental information, signal the information via the secretion of hormones, after which hormone receptors cause a concomitant change in immune response (Lessells 2008). Further fieldwork is required in order to determine the mechanism by which *A. planci* detects local population density and consequently upregulates its immune response.

In conclusion, this study highlights density-dependent prophylaxis in a population of the echinoderm *A. planci*. This is the first demonstration of the density-dependent prophylaxis hypothesis outside arthropods. The presence of phenotypic plasticity for immune response may go some way to explain both the persistence of *A. planci* populations during an outbreak and their relatively long duration before collapse and disease is unlikely to be the reason for their population demise. *Acanthaster planci* outbreaks result in marked decreases in live coral cover (Bruno and Selig 2007), with severe ecological consequences for individual reefs. However, biological control via the introduction of a novel disease is unlikely to be an effective method in reducing their numbers.

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