Basal superoxide as a sex-specific immune constraint

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There is increasing evidence that reactive oxygen species (ROS), a group of unstable and highly reactive chemical molecules, play a key role in regulating and maintaining life-history trade-offs. Upregulation of ROS in association with immune activation is costly because it may result in an imbalance between pro- and antioxidants and, hence, oxidative damage. Previous research aimed at quantifying this cost has mostly focused on changes in the pro-/anti-oxidant balance subsequent to an immune response. Here, we test the hypothesis that systemic ROS may constrain immune activation. We show that systemic, pre-challenge superoxide (SO) levels are negatively related to the strength of the subsequent immune response towards the mitogen phytohaemagglutinin in male, but not female painted dragon lizards (*Ctenophorus pictus*). We therefore suggest that systemic SO constrains immune activation in painted dragon males. We speculate that this may be due to sex-specific selection pressures on immune investment.

Keywords: reactive oxygen species; immunity; *Ctenophorus pictus*; phytohaemagglutinin

1. INTRODUCTION

Reactive oxygen species (ROS), a group of unstable and highly reactive chemical molecules, have recently become an important focus in evolutionary ecology research. There is increasing evidence that ROS are involved in physiological processes which link life-history traits, such as reproduction and ageing and, hence, play a key role in regulating and maintaining life-history trade-offs (e.g. [1,2] for comprehensive reviews).

While respiration, i.e. the reduction of molecular oxygen to form water by the electron transport chain in the mitochondria, is the major source of ROS production in aerobic organisms [3], ROS-producing enzymes and enzyme complexes involved in immune defence constitute other important sources of ROS production [1,2]. Among those, the NADPH oxidase enzyme complex, which is located in the membranes of professional phagocytes (e.g. macrophages) as well as B and T lymphocytes, is thought to play a major role in this type of ROS production [4]. Oxidative

burst, the rapid production of high cellular ROS levels through NADPH oxidase, is an integrated part of the innate immune system with the function to kill invading bacteria and other microbes [4].

Although ROS fulfil important functions in cellular signalling (e.g. as modifiers of signalling pathways that control the proper development and proliferation of cells [5]) as well as in killing foreign pathogens (see above), overproduction of ROS can inflict oxidative damage on cell components. To minimize such cellular damage by ROS, aerobic organisms possess a sophisticated system of endogenous antioxidant defences, which scavenge unwanted ROS [4]. The depletion of antioxidant reserves by immune system generated ROS is thought to represent a significant cost of immune activation with potentially important fitness consequences (e.g. [6,7]).

However, the cost of immune activation in terms of immune-generated ROS is unlikely to depend solely on the availability of antioxidant defences. Here, we explore the possibility that systemic ROS levels may act as constraints for immune function. Studies to date have mainly considered changes in the pro-/anti-oxidant balance subsequent to an immune challenge (e.g. [7] and references therein). However, because immune activation is associated with an increase in ROS levels, we hypothesize that individuals with high inherent ROS profiles may be limited in their capacity to mount an immune response as they may be more prone to the risk of oxidative damage. We tested this hypothesis in wild-caught painted dragon lizards (*Ctenophorus pictus*) by measuring cellular ROS levels and relating them to the subsequent immune response to the mitogen phytohaemagglutinin-A (PHA). We predicted that if systemic ROS levels compromise immune function then the strength of the immune response should be negatively related to ROS levels.

2. MATERIAL AND METHODS

(a) *Animal husbandry*
The lizards were caught at Yathong Nature Reserve, New South Wales (145°35′; 32°35′) at the beginning of the mating season (September 2008) and brought back to holding facilities at Wollongong University. All lizards were kept individually in cages (60 × 60 × 50 cm), on a 12 L : 12 D regime, with a spotlight at one end of the cage to allow thermoregulation to the preferred body temperature and fed crickets and meal worms every second day. The experiment as described below was conducted after one month in captivity. Sample sizes were $n = 20$ for males and $n = 25$ for females.

(b) *Quantifying reactive oxygen species levels*
All lizards were blood-sampled prior to immunization with PHA (see below). Blood was collected with a glass capillary from *vena angularis* (in the corner of the mouth). Systemic ROS levels were quantified according to previously specified protocols [8]. Briefly, we used flow cytometry in combination with two probes (MitoSOX Red (MR) and dihydrorhodamine 123 (DHR), Invitrogen) that freely diffuse into cells, accumulate within the mitochondria and become fluorescent when oxidized by specific ROS (MR measures specifically superoxide (SO); DHR identifies various ROS species, including singlet oxygen, SO, H_2O_2 and peroxynitrite, hereafter, referred to as 'unspecific ROS'). Details of flow cytometry methods are included in the electronic supplementary material.

(c) *Phytohaemagglutinin challenge*
To assess whether circulating ROS levels constrain immunity, we used a PHA assay. After blood-sampling, lizards were injected with 30 µg PHA (Sigma L-8754) dissolved in 30 µl sterile phosphate-buffered saline (PBS) into the left hindfoot pad. PHA injection produces local inflammation and swelling and can therefore be used a simple immune measure to assess an individuals' ability to mount an inflammatory response [9]. The same volume of PBS only was

injected into the right hindfoot pad as a control. The thickness of each foot pad was measured three times to the nearest 0.01 mm with digital calipers immediately before and 24 h (± 0.5) after injection. The mean of the three measures was used in analyses. The strength of the immune response was assessed as the difference in swelling between the PHA-injected and the control foot pads. Concomitant with the measures of foot pad thickness, lizards were weighed to the nearest 0.01 g and their snout–vent–length (SVL) measured to the nearest 0.5 mm.

(d) Statistical analyses

Statistical analyses were performed in SAS System v. 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). We used general linear models (PROC GLM) with immune response or body mass change as dependent variable, sex as a fixed factor, and ROS levels (basal SO (bSO) and unspecific ROS) and SVL as covariates. SVL was included in the analysis because painted dragons are sexually dimorphic (with males being larger than females) and because SVL is a proxy of age (being ectotherms, painted dragons grow throughout life). Two-way interactions between factors and covariates were initially included in the models, but removed at $\alpha > 0.25$ [10]. Residuals of the models were tested for normality. All tests were two-tailed with a significance level set at $\alpha < 0.05$.

3. RESULTS

There was no sex difference in bSO or unspecific ROS levels ($p > 0.25$). bSO levels were negatively related to SVL ($F_{1,43} = 5.21$, $p = 0.028$), but not those for unspecific ROS ($F_{1,43} = 0.10$, $p = 0.76$). Males had on average lower immune responses than females (least-squares means; 0.27 ± 0.06 mm versus 0.57 ± 0.05 mm, (mean $\pm$ s.e.)) and larger individuals mounted stronger immune responses than smaller ones (table 1). The strength of the PHA response was significantly predicted by bSO levels at the time of immunization, but in a sex-dependent manner (sex by bSO interaction; table 1). The strength of the immune response was negatively related to bSO levels in males, but not in females (figure 1). Unspecific ROS were not related to the strength of the immune response ($p > 0.39$ for main effect and its interaction with sex).

Body mass change within 24 h after PHA injection was not affected by bSO or unspecific ROS levels ($p > 0.25$ for main effects and interactions with sex). Sex also did not affect body mass change ($F_{1,41} = 0.24$, $p = 0.63$). Moreover, body mass change was not related to the strength of the immune response (body mass change: $F_{1,36} = 1.36$, $p = 0.25$; body mass change by sex interaction: $F_{1,41} = 0.05$, $p = 0.82$). There was, however, a significant negative relationship between SVL and change in body mass (b: -0.02 ± 0.01, $F_{1,43} = 4.09$, $p = 0.049$). Larger individuals lost weight 24 h post immunization whereas smaller ones gained weight.

4. DISCUSSION

As predicted, we found a negative relationship between systemic ROS levels and the strength of the immune response. The relationship was specific to bSO, but not unspecific ROS and was restricted to males only. We have previously shown that bSO levels, but not unspecific ROS levels, are heritable [11] suggesting that individual variation in lizard SO levels may be less plastic in response to environmental variation. This is corroborated by a previous study in which we show that bSO, but not unspecific ROS, are negatively correlated with colour change in male painted dragons [9]. Furthermore, our
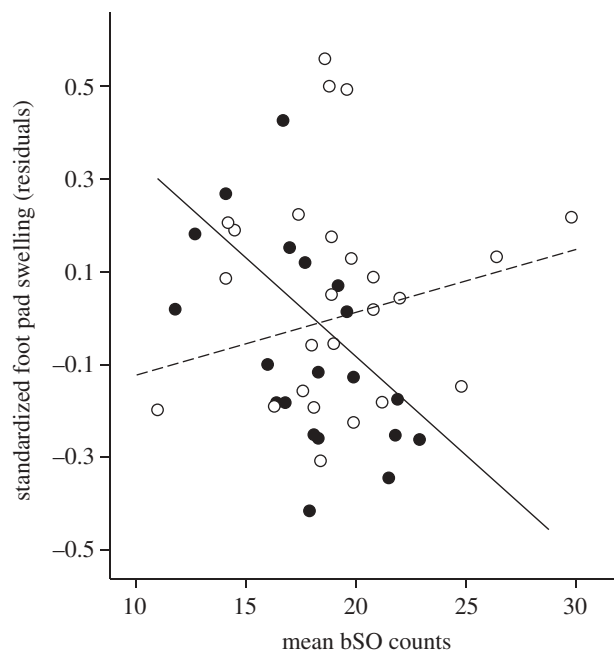

Figure 1. Scattergram illustrating the significant interaction term between sex and basal superoxide (bSO) levels for the immune response towards PHA (standardized for body size; residuals from a foot pad swelling—SVL regression). Males, filled circles and solid line ($n = 20$; b: -0.043 ± 0.014; $F_{1,18} = 9.10$, $p = 0.007$); females, open circles and dashed line ($n = 25$; b: 0.014 ± 0.012; $F_{1,23} = 1.37$, $p = 0.25$).

Table 1. Effects of sex, body size (snout–vent–length = SVL) and basal superoxide (bSO) on PHA-induced swelling in the hindfoot of painted dragon lizards (see §2 for details).

source	d.f.	SS	MS	F	p
response variable: PHA-induced swelling (model $F_{4,40} = 4.53$, $p = 0.004$, $r^2 = 0.31$)					
sex	1	0.233746	0.233746	5.45	0.025
bSO	1	0.076642	0.076642	1.79	0.189
bSO × sex	1	0.391690	0.391690	9.14	0.004
SVL	1	0.460213	0.460213	10.74	0.002

results are in line with recent studies on mutant mice which exhibit reduced/elevated mitochondrial SO production and associated enhancement/impairment of immune function [12,13].

Why was the negative relationship between SO and immunity restricted to males? Female painted dragons showed on average a stronger immune response towards PHA than males, which is consistent with higher female immuno-competence in many vertebrate species (e.g. [14] for a recent review). Bateman's principle predicts that males maximize fitness through an increase in mating rate whereas females maximize fitness through investment in longevity [15], and therefore, selection should favour enhanced immunity in females [16]. Moreover, a recent study on tarantula spiders (*Brachypelma albipilosa*) suggests that sex-specific differences in longevity are associated with differences in several ROS parameters, one of them being mitochondrial SO production [17]. Male and female painted dragons differ in many behavioural and reproductive characteristics. Males engage in

overt aggressive behaviour with rising testosterone levels and open exposure to rivals and predators during territory patrolling and mate acquisition throughout the day [18,19]. Females have a more cryptic lifestyle and spend considerable time basking to maintain appropriate incubation conditions for developing follicles and eggs [19]. These differences are likely to be reflected in physiological parameters. SO production is linked to metabolic rate and, hence, energetic requirements. It is possible that the negative relationship between bSO and immune responses in males reflects sex-specific energy requirements. However, if the relationship were purely dependent on energetic demands then a negative relationship between the change in body mass and the immune response might be expected. Alternatively, we suggest that different sources of ROS production (i.e. respiration and 'ROS-producing' enzyme complexes involved in immune activation) may negatively affect each other and that males with higher systemic SO levels mount smaller immune responses because they may be limited in their ability to mobilize antioxidant defences in response to the immune activation. In accordance with Bateman's principle, we speculate that higher immune responses in painted dragon females, and the lack of a negative relationship between ROS and the strength of the immune response, in particular, may be owing to sex-specific selection pressures on immune investment.

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