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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

Contrasted effects of an oxidative challenge and α-melanocyte-stimulating hormone on cellular immune responsiveness: an experiment with red-legged partridges *Alectoris rufa*

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Abstract Oxidative stress is increasingly recognized as a key selective force shaping evolutionary trade-offs. One such trade-off involves investing in immunity versus combating oxidative stress. While there is broad evidence that mounting an immune response causes increased oxidative stress, the effect that increased oxidative stress during development has at a later stage on immune responsiveness remains little known. The production of melanin-based coloration in vertebrates is influenced by oxidative stress and by hormones, such as the alpha-melanocyte-stimulating hormone (α-MSH). Oxidative stress could impair immunity, and this might be a cost associated with the production of melanin traits. α-MSH has immunomodulatory effects, with most evidence pointing towards an improvement of immunity (improved pro-inflammatory activity). Here, we investigated the effects of an oxidative challenge (exposure to a pro-oxidant compound, diquat) and of experimentally elevated α-MSH on the cell-mediated immune responses (CMIR) of growing young (1 month old) red-legged partridges *Alectoris rufa* in captivity. CMIR were assessed in response to primary and secondary challenges with phytohemagglutinin (PHA). We specifically tested whether an oxidative challenge during growth and development had a delayed effect (4 months after exposure) on immunity. We found that the diquat treatment did not affect primary CMIR, but significantly reduced secondary CMIR. Elevated α-MSH increased primary CMIR in males, but not in females. Our experimental results are consistent with a trade-off between investing in activities that generate oxidative stress (e.g., growth, reproduction, production of ornaments) versus investing in immunity, and shed new lights onto the inter-relationships between immunity, oxidative stress and the expression of melanin-based coloration in vertebrates, revealing a novel, delayed physiological cost that can contribute to ensuring honest signaling.

Keywords Acquired immunity · Diquat · Innate immunity · Melanin-based signaling · Oxidative stress · Ornament · Phytohaemagglutinin skin test

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Introduction

Environmental conditions during growth and development can have delayed effects on adult phenotypes (Metcalfe and Monaghan 2001). For instance, environmentally induced accelerated growth was recently associated with a reduced resistance to oxidative stress (Alonso-Alvarez

et al. 2007; Monaghan et al. 2009). Oxidative stress is often defined as the imbalance between the rate of production of reactive oxygen and nitrogen species (ROS and RNS, respectively) by the cell metabolism and the state of the repair and antioxidant machineries (Halliwell and Gutteridge 2007). ROS/RNS are molecules produced by oxidation and reduction reactions, and are unstable, very reactive by-products of normal metabolic processes inducing extensive damage to cellular proteins, lipids and DNA, particularly when individuals lack sufficient antioxidant protection (von Schantz et al. 1999; Halliwell and Gutteridge 2007). However, the effect that a high exposure to ROS/RNS during development imposes on the later performance of some vital processes, such as the immune function, remains little known.

There is growing and broad evidence that mounting an immune response against parasites and pathogens leads to increased oxidative stress (reviewed in Sorci and Faivre 2009). Immune cells such as macrophages and neutrophils (heterophils in birds) produce ROS/RNS to destroy the invading pathogens (Swindle and Metcalfe 2007; Sorci and Faivre 2009), but the toxicity of these compounds may not be restricted to infectious organisms, and the overproduction of ROS/RNS can produce oxidative damage (Finkel and Holbrook 2000; Halliwell and Gutteridge 2007). In contrast, the impact that elevated oxidative stress may exert on immune function is less well understood (reviews in Kurien and Scofield 2008). Oxidative stress could potentially damage both the innate and acquired components of the immune system, and can also stimulate antibody production leading to autoimmune reactions (Kurien and Scofield 2008).

Amongst cells involved in immune defence, T lymphocytes (T cells) appear particularly sensitive to ROS. In mammals and birds, T cells participate in both innate and acquired immune responses (Martin et al. 2006; Murphy et al. 2007; Tella et al. 2008). Different T cell lineages are involved in the activation of phagocytes responsible for inflammatory burst and ROS release for pathogen destruction (e.g., Murphy et al. 2007). T cells also activate B lymphocytes (B cells) to produce antibodies, whereas specific memory T cells are able to mount a faster and stronger immune response when the organism is re-exposed to the same infectious agent (e.g., Murphy et al. 2007). Studies in mammals have revealed that T cell production depends on intracellular antioxidant levels, with oxidative stress being associated with higher rates of T cell death (i.e. apoptosis; Meier and Vousden 2007). Therefore, since immune and antioxidant systems may compete for the same resources (e.g., antioxidant molecules such as glutathione), it has been hypothesized that a trade-off may exist between both traits (e.g., Horak et al. 2007; Monaghan et al. 2009). Thus, it can be predicted that situations involving high oxidative stress should compromise the capacity of an individual to mount an effective immune response. To date, few studies have tested the impact of elevated oxidative stress on the immune function of vertebrates (e.g., Salazar-Lugo et al. 2009; Deng et al. 2010), and even fewer studies have experimentally caused oxidative challenges in a direct in vivo manner (Isaksson and Andersson 2008; Galván and Alonso-Alvarez 2008, 2009; Horak et al. 2010).

In addition to factors promoting oxidative stress, physiological endogenous variables also determine the capacity to develop an efficient immune system. Steroid hormones, such as corticoids and testosterone, have the potential to down-regulate immune responses (e.g., Bortolotti et al. 2009), although this may depend on species (Roberts et al. 2004). However, the possible effects that other hormones may exert on the regulation of immune defenses remain less well understood. For instance, the α-melanocyte-stimulating hormone (α-MSH) has immunomodulatory effects on the innate branch of the immune response of vertebrates and also regulates the synthesis of melanin (e.g., Lin and Fisher 2007; Ducrest et al. 2008). The production of melanin is one of the functions of this hormone, determining the expression of melanin-based traits (e.g., Höhn and Braun 1980; Bowers et al. 1997; Ducrest et al. 2008; Galván and Alonso-Alvarez 2009), which are amongst the commonest colored traits and conspicuous ornaments of vertebrates (McGraw 2006). These melanin traits can have utilitarian functions such as camouflage or quality advertisement (Roulin et al. 2000; McGraw 2006; Meunier et al. 2011), with functions being most likely trait- and species-specific (Meunier et al. 2011). Here we focus on melanin-based traits that are used in social interactions and may advertise an individual's quality, such as a better immunocompetence (e.g., Roulin et al. 2000). A positive association between the expression of a melanin-based trait and immunocompetence might arise because hormones regulating the production of melanin, such as α-MSH, also enhance immune responsiveness (review in Brzoska et al. 2008; Ducrest et al. 2008). An alternative hypothesis previously proposed that maintaining elevated levels of α-MSH may depress, not enhance, immunity (review in Moreno and Møller 2006). If so, it would provide a cost (or handicap; sensu Zahavi 1975) associated with trait production, which could ensure honest sexual signaling. Such a possible cost associated with the production of melanin-based ornaments (associated with an immunosuppressant activity of α-MSH) has, however, yet to be demonstrated for birds.

Here, we studied the impact that both an oxidative challenge and α-MSH levels during growth may exert on the immune system after the developing period, using the red-legged partridge *Alectoris rufa* as a model species. To

measure immune responsiveness, the birds were subcutaneously inoculated with a mitogen (i.e. lectin, a phytohaemagglutinin; PHA) that provokes an inflammatory reaction. This technique is amongst the most accepted and used in ecological studies, because it is well validated (e.g., Martin et al. 2006; Tella et al. 2008). Recent works have highlighted that the PHA skin test, in birds, allows measuring aspects of innate (non-specific) immunity (involving basophiles, heterophiles and macrophages; Martin et al. 2006; Tella et al. 2008). More specifically, the response to a primary injection most likely reliably mirrors an overall individual's pro-inflamatory potential (Vinkler et al. 2010), while the response to a second injection allows measuring the intensity of the acquired component of the immune response (Janeway and Travers 1999; Tella et al. 2008). In fact, the second exposure to the same mitogen (PHA) induces a stronger inflammatory response in birds revealing the activation of memory T cells and specific antibodies (Tella et al. 2008).

We evaluated the single and interactive effects of an oxidative challenge (by supplying a low dose of diquat, a free radical generator, in drinking water) and elevated α-MSH levels (by intramuscular injections) on primary and secondary cellular immune responses, using a factorial experimental design. The mechanism of action of diquat is via the formation of free radicals when the molecule is broken, i.e. catabolized (Stancliffe and Pirie 1971). In previous works with red-legged partridges, it was reported that the same dose of diquat is able to increase the amount of lipid peroxidation in erythrocytes by 20% after 15 days of exposure (see pilot study reported in Alonso-Alvarez and Galván 2011), but also to reduce it after 33 days (Galván and Alonso-Alvarez 2009). In the latter study, it is also reported that the diquat exposure depleted antioxidant levels (i.e. erythrocyte glutathione levels) after 33 days, consistent with the diquat having caused an oxidative challenge (Galván and Alonso-Alvarez 2009). Both the diquat and α-MSH treatments enhanced eumelanin-based (i.e. the darkest form of melanin) ornamentation (diquat alone doubled black bib size, and α-MSH alone caused a 50% increase in bib size of red-legged partridges; see Galván and Alonso-Alvarez 2009). The diquat treatment alone may have increased bib size because it reduced glutathione levels (an intracellular antioxidant depleted during situations of increased oxidative stress), and because higher levels of glutathione inhibit eumelanin production (see Galván and Alonso-Alvarez 2008, 2009).

Here, we report on the delayed effects of these treatments on cellular immune responsiveness using the same experimental birds as in Galván and Alonso-Alvarez (2009). Because young partridges were treated during their growth, when still developing their immune system (Apanius 1998; Glick 2000), we expected long-lasting effects on immune responsiveness. We predicted that the diquat treatment (oxidative challenge) would compromise the capacity of an individual to mount an effective immune response, reducing primary and/or secondary inflammatory and immune responses to PHA. Given the known immunomodulatory effects of α-MSH (Brzoska et al. 2008; Ducrest et al. 2008), high levels of this hormones could have delayed positive effects on responses to PHA. Conversely, the handicap hypothesis would predict that partridges exposed to α-MSH during growth could develop a reduced capacity to mount primary and/or secondary immune responses (Moreno and Møller 2006). The effects of diquat and α-MSH might be additive (birds simultaneously exposed to diquat and α-MSH could develop the worst immune capacity) or compensatory (high α-MSH levels can increase resistance to oxidative stress; Ducrest et al. 2008). If the impact of these factors is stronger on T cell-mediated pathways, secondary responses should be more impaired than primary immune response.

Materials and methods

Experimental design

We carried out the experiment in July–October 2008 at the Dehesa Galiana experimental facility (Ciudad Real, Spain), using 78 young red-legged partridges, all born in captivity in summer 2008. When about 20 days old, the partridges were marked with a numbered metal ring, randomly assigned to one of four treatments (following a factorial experimental design: control, α-MSH, diquat, or α-MSH and diquat; Galván and Alonso-Alvarez 2009), and transferred to eight different indoor aviaries (each 4 × 3 m and 3 m high; light regime: 13:11 h, L:D; ad libitum pelleted food). Birds from different treatment groups were held in different aviaries (see below). The number of birds per aviary averaged 9.6 ± 1.2 (range 8–11) with food being provided ad libitum in each aviary throughout the study. The identity of the parents of each bird (all were born in captivity) was randomized among experimental groups (see Galván and Alonso-Alvarez 2009).

Birds treated with diquat were supplied diquat dibromide in drinking water in aviaries corresponding to this treatment (diquat-treated partridges). Diquat is commonly used as an aquatic herbicide whose mechanism of action is the production of reactive oxygen species, particularly intracellular superoxide anion (e.g., Sewalk et al. 2001; Zeman et al. 2005; Xu et al. 2007). The commercial product ('Reglone'; Syngenta, Madrid) consisted in 20% w/v of diquat dibromide in water (consultation with the company). The treatment with diquat in water lasted 33 days (Table 1), with a dose of 0.5 ml diquat per liter of

Table 1 Timing of the experiment, procedures and data sampling

Sampling time	S0	S1	S2
Dates	8–23/06/2008	27–28/10/2008	18–19/11/2008
Treatment	α-MSH (injections during 15 days)		
	Diquat (in drinking water for 30 days)		
Assessment of cellular immune responses		First PHA injection (CMI 1)	Second PHA injection (CMI 2)

water, which was calculated from a pilot study (see Alonso-Alvarez and Galván 2011). In that pilot study, birds were exposed to five different diquat doses during 15 days. The intermediate dose (0.5 ml/l) induced higher lipid peroxydation levels in blood compared to controls, without birds losing body mass or suffering increased mortality. Each aviary contained a tank with 4 l of water, which was replaced every 4 days to avoid the disintegration of the diquat molecule (i.e. following product properties; Syngenta).

Birds treated with the melanocyte-stimulating hormone were administered α-MSH (Sigma, St. Louis, USA) by means of intramuscular injections in the left pectoral muscle at a dose of 0.04 mg of α-MSH dissolved in 2 ml of phosphate buffered saline (PBS) per bird. Control birds received injections of 2 ml of PBS only. The injection site was sterilized with alcohol, and injections were administered every 2 days during a 15-day period (i.e. 8 injections per bird; Table 1). Overall, each partridge received a total amount of 0.32 mg of α-MSH. This dose was adjusted according to the average initial mass (200 g) of the red-legged partridges in the study, so that it was equivalent to that used by Höhn and Braun (1980) on rock ptarmigan *Lagopus mutus* (total dose of 0.0016 mg of α-MSH per g). Doses were not adjusted to the individual mass of each partridge, but were the same for all experimental birds.

Assessment of cellular immune responses

In order to measure primary and secondary immune responses, we conducted PHA skin tests on each bird twice (on 27–28/10/2008 = S1 and on 18–19/11/2008 = S2; Table 1). The role of cellular immunity in combating particular parasites and pathogens is, alone, not enough to explain many immunological patterns and processes (Owen and Clayton 2007). Nevertheless, the assessment of this immune component remains one of the most often used technique in ecological immunology, particularly in avian studies (Martin et al. 2006; Tella et al. 2008). For each test, partridges were injected subcutaneously in the left wing patagium with 0.5 mg of PHA (Sigma-Aldrich; ref. L-8,754) suspended in 0.1 ml of PBS (PHA-group), following Smits et al. (1999). This concentration was similar to that used in previous PHA tests conducted on this species (e.g., Mougeot et al. 2009a; Perez-Rodriguez et al. 2008, 2010) and was the same for all individuals (PHA doses were not adjusted to the mass of each individual partridge). Before injection, the thickness of the patagium was measured with a digital spessimeter (Mitutoyo Absolute 547-315; Kawasaki, Kanagawa, Japan) to the nearest 0.01 mm. After 24 h, patagium thickness was again measured at the point of injection and the difference between initial and final measurements in PHA-injected birds was used as a proxy for the strength of cell-mediated immune response (Smits et al. 1999). In both cases (before injection and 24 h after injection), three measures of patagium thickness were taken by the same person (F.M.). Both initial and final measurements were repeatable (repeatability values calculated following Lessells and Boag 1987 at 0.95 and 0.93, respectively; both $P < 0.001$), and therefore we used average values of the three measurements in the analyses. The same procedure was followed to measure primary and secondary immune responses, but injections were done on the left patagium for the first test, and on the right patagium for the second test (as in Tella et al. 2008), to avoid possible effects of the previous injection. PHA responses could not always be measured on all birds. No PHA responses were measured for instance when a bird moved during patagium thickness measurements or injection, when a bird wounded itself during transport, or escaped. Hence, from an initial sample size of 78 birds, we obtained reliable immune response measurements from 71 birds (S1, first challenge) and 65 birds (S2, second challenge).

Measurements

Upon S1 and S2 (Table 1), we also measured tarsus length (with a calliper, nearest 0.1 mm) and weighed each bird (with an electronic balance, nearest 1 g). We calculated the body "condition" index of body mass corrected for size (hereafter "body mass index"), using the residuals from a GLM of log-transformed body mass on log-transformed tarsus length, as an index of structural size, similar to previous work with this species (Bortolotti et al. 2006; Mougeot et al. 2009a).

Molecular sexing

Partridges were sexed genetically from an aliquot of blood cell fraction. DNA from sex chromosomes was amplified by PCR (primers 2550F and 2718R; Griffiths et al. 1998).

Statistical analyses

We used SAS for all analyses (SAS 2001). Primary (CMI 1) and secondary (CMI 2) immune responses (changes in web swelling) were fitted to models using a normal error distribution (their distribution did not differ from a normal distribution; Wilk–Shapiro tests). All models included the identity of the parents (i.e. the identity of the couple that laid the egg) and the aviary in which the partridges were held during experiment as random factors (MIXED procedure; SAS 2001). Random factors were always maintained in the models, although similar results were obtained when removed. When testing for experimental effects on study parameters, initial models included sex, diquat treatment, α-MSH treatment and all the interactions between these factors as fixed effects. Starting with these models, a backward stepwise procedure was used, removing non-significant terms (at the $P = 0.10$ levels) starting with interactions. We kept significant terms ($P < 0.05$ levels) as well as marginally significant effects ($0.05 < P < 0.10$) worth reporting. The Satterthwaite correction was used to approximate the degrees of freedom. We show the results of the full models, as well as those of the reduced models, but parameter estimates only for the reduced models (those with only significant terms or interactions). When analyzing variation in cell-mediated immune responses (CMI 1 and CMI 2), we included body mass index (at S1 and S2, for CMI 1 and CMI 2, respectively) as a covariate, because immune responses are often body mass index-dependent, as previously shown for red-legged partridges (e.g., Mougeot et al. 2009a). When analyzing variation in CMI 2, we also included the CMI 1 as a covariate, to control for differences in primary responses. We calculated changes in immune responses between primary and secondary responses as the absolute difference corrected for the initial response. All tests are two-tailed, and all data are expressed as means ± SEM.

Results

Effects of diquat, α-MSH and their interaction on primary immune responses

Variation in primary immune responses was significantly explained by the body mass index at S1 and by the α-MSH × sex interaction, but not by diquat, either as a single factor or in interaction with sex or α-MSH (Table 2). Primary responses positively correlated with body mass

Table 2 Results of the GLMs testing for treatment effects on primary (CMI 1) and secondary (CMI 2) cellular immune responses in red-legged partridge *Alectoris rufa* (see "Materials and methods")

Dependent variable	Fixed effects	Initial model			Reduced model			Parameter estimate ± SEM
		df	*F*	*P*	*df*	*F*	*P*	
Primary response (CMI 1)	Body mass index at S1	1.53	7.36	<0.01	1.58	9.12	<0.01	Intercept +1.824 ± 0.086 Condition +1.637 ± 0.557
	MSH	1.53	1.56	0.217	1.58	1.32	0.256	No α-MSH −0.314 ± 0.109
	Diquat	1.53	0.78	0.381				
	Diquat × MSH	1.53	0.01	0.929				
	Sex	1.53	1.63	0.207	1.58	0.37	0.544	Female −0.205 ± 0.115
	MSH × sex	1.53	7.24	<0.01	1.58	8.62	<0.01	Female × No α-MSH +0.447 ± 0.152
	Diquat × sex	1.53	0.22	0.643				
	Diquat × MSH × sex	1.53	0.03	0.866				
Secondary response (CMI 2)	Primary CMI	1.47	2.52	0.119	1.54	3.47	0.068	Intercept +1.116 ± 0.250 CMI 1 +0.241 ± 0.138
	Body mass index at S2	1.47	0.45	0.506				
	MSH	1.47	0.08	0.777				
	Diquat	1.47	6.76	<0.05	1.54	7.03	<0.05	No diquat +0.235 ± 0.108
	Diquat × MSH	1.47	0.01	0.909				
	Sex	1.47	2.58	0.115	1.54	3.77	0.058	Female +0.166 ± 0.099
	MSH × sex	1.47	0.62	0.437				
	Diquat × sex	1.47	0.91	0.346				
	Diquat × MSH × sex	1.47	0.09	0.766				

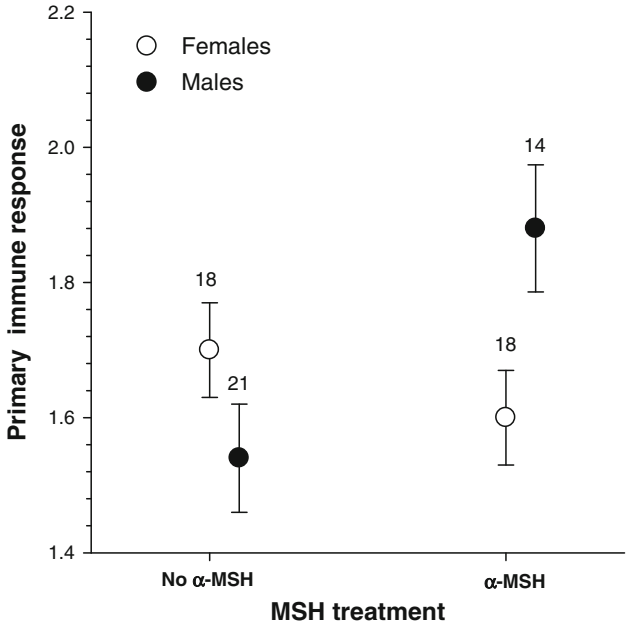

Fig. 1 Mean ± SEM primary cellular immune responses in red-legged partridge *Alectoris rufa* (wing web swelling 24 h post-PHA injection, in mm) according to sex (*open symbols* females, *filled symbols* males) and α-MSH treatment (0 = control, 1 = treated). Sample size above *bars* refers to number of birds

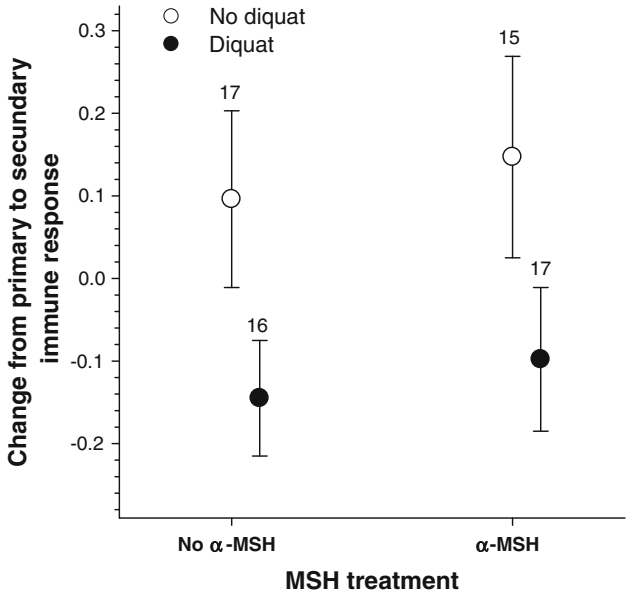

Fig. 2 Mean ± SEM change in immune responsiveness (wing web swelling 24 h post-PHA injection) between the first and the second PHA injection according to treatment (*open symbols* no diquat, *filled symbols* diquat-treated; *x*-axis: 0 = no α-MSH, 1 = α-MSH treated). Sample size above *bars* refers to number of birds

index at S1 (Table 2; slope ± SEM: +1.637 ± 0.557). In addition, the α-MSH treatment increased primary responses, but only in males ($F_{1,25} = 6.38$; $P = 0.018$; estimate ± SEM for α-MSH effect: +0.303 ± 0.120), not in females ($F_{1,25} = 1.51$; $P = 0.230$; estimate for α-MSH effect: −0.121 ± 0.098) (Fig. 1).

Effects of diquat, α-MSH and their interaction on secondary immune responses

Variation in secondary immune responses was significantly explained the diquat treatment, and marginally explained by primary responses (CMI 1), by sex, but not by the body mass index at S2 or the α-MSH treatment, either as a single factor or in interaction with sex or diquat treatment (Table 2). The significance of the diquat treatment effect was maintained when the marginally significant terms (sex and primary response) were removed from the model (diquat: $F_{1,56} = 6.24$; $P < 0.05$). Secondary responses were overall greater than primary responses (mean ± SEM, CMI 1: 1.664 ± 0.041, $n = 71$; CMI 2: 1.731 ± 0.052, $n = 65$) and tended to be positively correlated with primary responses (slope ± SEM: +0.241 ± 0.138; Table 2). The relative increase in web swelling from primary to secondary immune response was independent of body mass index at S2 (Table 2), tended to be greater for males than for females (Table 2; mean change ± SEM, males: +0.086 ± 0.064, $n = 33$; females: −0.089 ± 0.077, $n = 32$), but was significantly lower in birds treated with diquat than in birds not treated with diquat, in both sexes and irrespective of α-MSH treatment (Table 2; Fig. 2). In partridges previously exposed to diquat, secondary responses to PHA (wing web swelling, in mm) averaged 1.856 ± 0.084 ($n = 32$) in birds not treated with diquat, and 1.608 ± 0.055 ($n = 33$) in diquat treated birds.

Discussion

Our results show that an exogenous oxidative challenge (free radical exposure) during development constrains the capacity of individuals to mount a secondary cell-mediated immune response. In contrast, α-MSH enhanced primary cell-mediated immune responses, but only in male partridges. Below, we discuss these findings and integrate them in a context of honest melanin-based signaling of individual quality.

Oxidative challenge and immune responsiveness

When exposed to a free radical generator (diquat) during development, the same partridges used in the present experiment developed lower levels of a key intracellular antioxidant, glutathione, but also lower lipid peroxidation levels in blood than controls after 33 days of treatment (Galván and Alonso-Alvarez 2009). However, in a pilot study, a 20% increase in lipid peroxidation levels was

found in diquat-treated birds as compared with controls after 15 days of exposure (Alonso-Alvarez and Galván 2011). Taking into account the decline in antioxidant levels, we can suggest that our birds were exposed to an oxidative challenge that probably triggered a compensatory response later on (Dimova et al. 2008; Costantini et al. 2010). Here, we investigated whether such an oxidative challenge affected immune responsiveness, specifically the ability to raise a cell-mediated immune response. Diquat exposure during the development of red-legged partridges did not significantly affect the response to the first PHA challenge (CMI 1), which would represent an individual's pro-inflammatory potential (Vinkler et al. 2010) and aspects of the innate (non-specific) immunity involving cells different from T-lymphocytes (particularly basophiles, heterophils and macrophages; Martin et al. 2006; Tella et al. 2008). This primary response to PHA challenge was body mass index-dependent, that is, relatively heavier partridges raised greater responses, as usually found in birds (e.g., Alonso-Alvarez and Tella 2001; Mougeot et al. 2009a). However, diquat exposure affected the secondary response to PHA, when birds were challenged again with PHA ca. 1 month after the first immune insult. As in previous work on birds (i.e. Tella et al. 2008), a second exposure to the same mitogen induced a stronger inflammatory response. Birds that raised greater responses in the first injection still mounted relatively greater responses in the second one. However, when partridges had been exposed to diquat during development, immune responses did not increase between the first and the second challenge, unlike in birds that had not been exposed to diquat. The oxidative challenge therefore negatively impacted on some aspects of cellular immune responses, and may have affected the acquired component of the cellular immune response (sensu Janeway and Travers 1999; Tella et al. 2008).

Previous avian studies have reported an increase in oxidative stress associated with a PHA immune challenge (Horak et al. 2007), supporting the existence of an oxidative cost associated with the cell-mediated immune response (Costantini and Møller, 2009; Sorci and Faivre 2009). In red-legged partridges, PHA is also able to induce a decrease in circulating levels of carotenoids, but had no noticeable short-term effect on oxidative stress (no significant change in TBARS 24 h after PHA challenge; see Perez-Rodriguez et al. 2008). Parasite challenges and infections were also shown to cause oxidative damage in another gallinacean (the red grouse *Lagopus lagopus scoticus*; Mougeot et al. 2009b; 2010), consistent with the idea that raising an immune response increases oxidative stress. Our experimental results thus indicate that the immunity-oxidative stress interactions can be a two-way interaction: raising an immune response can cause oxidative stress and a situation of increased oxidative stress during growth and development can negatively impact on immunity later on (such as acquired cellular immunity). Our results are therefore consistent with a trade-off between combating oxidative stress and investing in immunity. Nonetheless, future experiments should additionally test effects on other branches of the complex immune system (e.g., humoral component; e.g., Gasparini et al. 2009).

It is also worth noting that the effect of the treatment was noticeable several months after the end of the exposure to diquat, revealing the capacity of free radical exposure to induce long-lasting effects on individual phenotype. This was probably because the innate immune system was still developing (Apanius 1998; Glick 2000) during the diquat exposure. As far as we know, no study has tested and reported a delayed effect of an oxidative challenge on the immune response in any species. Nonetheless, Sewalk et al. (2001) reported delayed effects on mallard *Anas plathyrhynchos* hatchlings whose eggs were treated with diquat upon day 4 or 21 of the incubation period, with apparent effects being detected more than 1 month after initial treatment, in terms of oxidative damage (lipid peroxidation).

α-Melanocyte stimulating hormone and immune responsiveness

Melanin-based traits are amongst the commonest colored traits and conspicuous ornaments of vertebrates (McGraw 2006). The main function of α-MSH is to enhance the production of melanin (Höhn and Braun 1980; Bowers et al. 1997; Ducrest et al. 2008). Accordingly, red-legged partridges treated with α-MSH during growth developed larger black spotted bibs and broader flank bands composed by eumelanin (Galván and Alonso-Alvarez 2009), which are melanin-based traits displayed during social interactions (Bortolotti et al. 2006). Nonetheless, we must be cautious in interpreting our results as we did not know what natural variability there is in α-MSH levels in wild red-legged partridges. The dose of α-MSH used here was based on a previously published work on several grouse species (ptarmigan; Höhn and Braun 1980), but was not measured post-injection.

Melanin pigments can be synthesized de novo by vertebrates, and the expression of most melanic traits is under an apparently tight genetic control, so it is unclear to what extent melanic traits could act as honest signals of changing individual quality, such as "condition" or body mass index (Ducrest et al. 2008; McGraw 2006). This is because any reliable signal needs to be costly to produce or maintain, and these costs are generated by environmental factors. Nevertheless, a growing number of studies show a high phenotypic plasticity in these traits: the expression of

melanin-based traits can have an important environmental component as derived from exogenous factors that are critical for the synthesis of melanin (i.e. exogenous oxidative stress and availability of melanin precursors; Poston et al. 2005; Jablonski and Chaplin 2010).

Our results did not support the hypothesis of Moreno and Møller (2006), which proposes that a possible cost associated with the production of melanin-based ornaments could be related to an immunosuppressant activity of α-MSH. If α-MSH compromises immunity, only those individuals that are of higher quality and better able to cope with this immunosuppressive effect of α-MSH could afford circulating more α-MSH, and exhibit the most developed melanin traits (Moreno and Møller 2006). Our experimental results are in fact consistent with a positive immunomodulatory role of α-MSH (Ducrest et al. 2008): α-MSH did not reduce, but enhanced the primary cellular immune responses of males. A recent work indicates that the response to a primary PHA injection mirrors pro-inflamatory potential (Vinkler et al. 2010), and some of the expected effects of elevated α-MSH include an increase in the induction of pro-inflamatory mediators, a reduction in the induction of anti-inflamatory mediators, and an increased resistance to oxidative stress (review in Ducrest et al. 2008). In birds, α-MSH seems to decrease the respiratory quotient (Tachibana et al. 2007), and could thereby reduce energy consumption and associated ROS/RNS production. If so, elevated α-MSH would reduce oxidative stress (e.g., Costantini 2008; but see Barja 2007; Hulbert et al. 2007), hence allowing stronger immune responses, as we found in male partridges. To assess this hypothesis, further avian studies should simultaneously assess immune responses and metabolic rates of birds exposed to the hormone. We also found that α-MSH had no delayed effects on primary PHA responses in females, in contrast with males. This difference might be because of sex-specific differences in sexual or stress hormone levels in allocation priorities or maintenance costs associated with plumage development (e.g., Roberts et al. 2004; Mougeot et al. 2005). These sexual differences in the effect of α-MSH on primary PHA responses would deserve further investigation, and the possibility that levels of α-MSH differ between sexes could be worth exploring.

Interestingly, in captive male red-legged partridges, a larger black bib predicts a greater primary cellular immune response to PHA (Mougeot and Perez-Rodriguez, unpublished data). A positive association between melanin-based ornamentation and immune responsiveness might therefore arise because α-MSH enhances both the expression of the trait (i.e. Galván and Alonso-Alvarez 2009) and some aspects of immunity, at least in males (this study). Our results as a whole do not support the "handicap hypothesis", at least in terms of a reduction of cellular immunity as a cost to maintaining elevated α-MSH levels, and in captive conditions in which birds are provided with food ad libitum. Cellular immunity is only one component of the complex immune system of vertebrates (Owen and Clayton 2007), so further studies would be needed to better evaluate the effects that this hormone may have on other aspects of bird immunity.

Integrating the results in a context of melanin-based signalling

There is growing evidence that melanin-based traits, despite a relatively tight genetic control (Ducrest et al. 2008), have an important environmental component, and can act as honest signals of quality (Galván and Alonso-Alvarez 2008, 2009). Accordingly, recent studies have found a positive correlation between the level of expression of eumelanic traits and some measures of resistance to oxidative stress (e.g., Roulin et al. 2011). A key issue remains to better evaluate the possible costs associated with the production and maintenance of such traits, since these costs would ensure honest signaling of individual quality. In contrast with α-MSH, an oxidative challenge (diquat exposure) during development can both enhance eumelanin-based ornament expression (i.e. Galván and Alonso-Alvarez 2009) and impair some aspects of immunity at a later stage, such as acquired cellular immune responses (this study). Interestingly, this cost (a suppression of the acquired component of the cellular immune response) was apparent long after the influence on the development of the trait (here, 4–5 months after initial treatment; Table 1), which decouples it from the time when the plumage ornament was produced. This delay is important because the plumage traits are expected to function as quality indicators long after they were produced. It has been recently proposed that one of the costs inherent in the production of eumelanin (black) ornaments would be the necessity of lowering levels of the antioxidant glutathione because it blocks eumelanin synthesis (Galván and Alonso-Alvarez 2008). Thus, only those individuals able to counteract the potential negative effects of low glutathione levels would be able to fully express the ornaments (Galván and Alonso-Alvarez 2008). Diquat, but not α-MSH, treatment induced low glutathione levels in the same partridges (Galván and Alonso-Alvarez 2009). The results of this study therefore ultimately suggest that one of the cost of combating an oxidative challenge (and of associated low glutathione titres) is a later impairment of acquired cellular immunity. These ideas should stimulate more experimental work designed to better understand the complex inter-relationships between oxidative stress, antioxidant levels, immunity, hormones such as α-MSH, and the expression of melanin-based traits.

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