Synergistic and antagonistic interaction between different branches of the immune system is related to melanin-based coloration in nestling tawny owls

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Running title: Interaction within the immune system

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Abstract

When exposed to parasites, hosts often mount energetically expensive immune responses, and this may alter resource allocation between competing life history traits including other components of the immune system. Here, we investigated whether a humoral immune challenge towards a vaccine reduces or enhances the cutaneous immune responses towards an injection of lipopolysaccharid (LPS, innate immunity) and phytohaemagglutinin (PHA, T-cell immunity) in nestling tawny owls in interaction with the degree of plumage melanin-based colouration. The humoral immune challenge enhanced the response to LPS similarly in differently coloured nestlings. In contrast, the same humoral immune challenge enhanced immune response to PHA in dark reddish melanic nestlings while reducing it in pale reddish melanic nestlings. Our results highlight that both antagonistic and synergistic interactions can take place among branches of immune system, and that the sign and magnitude of these interactions can vary with immune responses involved and the degree of melanin-based coloration.

Keywords: colour polymorphism, immunology, melanin-based coloration, trade-offs, vaccination.

Introduction

When exposed to parasites, hosts often mount energetically expensive immune responses (Sheldon & Verhulst, 1996), and the cost of immunity have been repeatedly demonstrated to alter resource allocation between competing life history traits such as growth (Martin, 2005) and reproduction (Bonneaud et al., 2003, Uller et al., 2006). Interestingly, Martin et al., (2006a) have recently shown that activation of one immune response negatively affect a second immune response. This study suggests that trade-offs can occur within the immune system itself and mounting a specific immune response may preclude the ability to mount another component of the immune system if these two immune responses rely on the same resources to be efficient (antagonistic interaction, Goüy de Bellocq et al., 2006, Martin et al., 2006a). However, the immune system is an interconnected system and reverse predictions can also be made (Janeway et al., 2005). A specific immune challenge may stimulate other components of the immune system if resisting the spread of parasites necessitates a battery of immune responses for which the benefits outweighs the costs (synergistic interactions, Zuk & Johnsen, 1998; Janeway et al., 2005). In agreement with this idea, chicken artificially selected for low antibody responses to sheep red blood cells (i.e. low humoral immune response) show also lower innate and T-cell mediated immune responses (Parmentier et al., 1998). This experimental result provides good evidence that synergistic interaction can take place among branches of immune system. Depending on immune responses involved, it is therefore possible to observe that a first immune challenge could repress (antagonistic) or prime (synergistic) the second one. Until now, little attention has been given to potential interactions (antagonistic vs. synergistic) among different immune responses in natural population (Zuk & Johnsen, 1998). In particular, genetic factors have not yet been investigated as potentially mediating interactions between immune parameters. This raises the interesting possibility that genotypes may react differently after a first immune

challenge. For instance, an immune challenge may stimulate subsequent immune responses in one set of genotypes, while it may suppress other immune responses in another set of genotypes. In this context, melanin-based coloration is a good candidate to investigate potential associations between genotypes and variation in resource allocation strategies between different immune responses. Indeed, melanin-based colors have commonly a strong underlying genetic basis (but see Griffith et al., 1999), and evidence is accumulating that genes coding for melanin pigmentation can pleiotropically regulate energy homeostasis and immune functions (Ducrest et al., 2008). In agreement with this, different studies reported covariations between the degree of melanin-based coloration and resource allocation towards immune responses (Roulin et al., 2000; Roulin et al., 2001; Galeotti & Sacchi, 2003; Bortolotti et al., 2006; Gasparini et al., 2009, Piault et al. in press). Thus, one hypothesis is that melanin pigmentation can be used as a phenotypic marker of genotypes and resource allocation strategies, including investments in immunity.

In the present study, we investigated the relationship between melanin-based coloration and resource allocation towards two different immune responses following a humoral immune challenge in nestling tawny owls. Melanin-based plumage coloration in the tawny owl vary continuously from dark to pale reddish melanic and is strongly heritable ($h^2 = 0.93$; Gasparini et al., 2009; see also Brommer et al., 2005). In a Swiss population, we exchanged eggs between pairs of nests in order to allocate randomly genotypes across environments and thus disentangle environmental from origin-related effects (i.e. genetic and pre-hatching maternal effect) on phenotypic traits. Twelve days after hatching we injected a vaccine in half of nestlings to induce a humoral immune response and in the other half we injected a saline solution as a control. Ten days later, we compared the cutaneous immune response against the mitogens lipopolysaccharid (LPS) and phytohaematogglutinin (PHA) between non-vaccinated nestlings and individuals having previously mounted a humoral immune response against the

vaccine. A first immune challenge may stimulate or repress the cutaneous immune response to LPS and PHA as compared to control. We used LPS and PHA because they are inducing innate and T-cell mediated immune responses, respectively (Parmentier et al., 1998; Smits et al., 1999; Tella et al., 2008). We therefore examined whether a humoral challenge prime or not two different immune responses, namely innate and T-cell mediated immune responses. Different interactions could be expected between these pairs of immune responses, an effect that may depend on the individual melanin-based coloration (Gasparini et al., 2009; Piault et al. in press). Because *a priori* predictions on the direction of interactions are difficult to do, we tested the hypothesis that interactions between immune challenges (i.e. synergistic vs. antagonistic) are colour-specific in this bird species without formulating specific predictions.

Materials and methods

Experimental procedure

Fieldwork was carried out in western Switzerland in 2005 in a woodland area of 911 km^2 where we fixed 366 nest-boxes on trees between November 2004 and February 2005. In March 2005, we visited nest-boxes to exchange complete clutches between 51 pairs of nests based on the criteria that clutches were laid on a similar date (Pearson correlation: $r = 0.88$, $P < 0.0001$) and counted a similar number of eggs ($r = 0.36$, $P = 0.008$). In this way, nestlings were all raised by foster parents and each nest contained nestlings of a single origin. Within pairs of nests used to cross-foster eggs, foster and biological parents did not resemble each other with respect to plumage coloration (female: $r = -0.04$, n = 51, $P = 0.81$, male: r = 0.18, n = 33, $P = 0.31$, we captured 81 males over 102 experimental nests and for 18 pairs of nests at least one of the two males was not captured), and thus we successfully randomized genotypes among environments. Given that plumage coloration is explained by origin-related factors and not by the rearing environment (Gasparini et al., 2009), in our experiment any covariation

between nestling coloration and immunity should result from pre-hatching maternal effects or genetic factors rather than from post-hatching parental effects. In our population in 2005, pairing was not significantly assortative with respect to coloration (r = 0.16, n = 81 pairs, P = 0.13). Using the 84 nests (over 102) for which at least one offspring hatched, we visited nest-boxes when nestlings were 11.5 days ± 2.9 days of age (mean ± SD, range: 2-19) to blood sample them with a heparinised capillary (Microvette CB 300 LH, Sarstedt, Switzerland). Immediately after, half of the brood was injected subcutaneously in the neck with 0.1 mL vaccine solution (n = 138 TETRAVAC-nestlings) (TETRAVAC© vaccine, Aventis Pasteur MSD, Switzerland) while the other half was injected with 0.1 mL of phosphate buffer saline (PBS, n = 144 control-nestlings). Our experimental design ensured that we created two groups of nestlings differing in the intensity of humoral immune stimulation (challenged vs. non-vaccinated). At the time of injection, TETRAVAC- and control-nestlings did not differ in terms of hatching date, body mass, wing length, tarsus length and coloration (mixed model ANOVA with nest as a random effect, all P-values > 0.75). Furthermore, male nestlings were vaccinated with TETRAVAC as often as female nestlings (χ^2_1 = 0.36, P = 0.55).

Ten days after vaccination, the 84 experimental nests were visited again to collect a second blood sample to quantify antibody production towards the vaccine. On the same day, nests were randomly allocated into two groups that did not differ in terms of hatching date, brood size, mean body mass, wing length, tarsus length and nestling coloration (Student's t-tests performed on means per nest, all P-values > 0.29). In the first group of nests (n = 44), all nestlings were injected subcutaneously in the wing web with 20 µg of polysaccharides (LPS, from degenerated cell walls of *Escherichia coli* 055:B5, Sigma, L2880, Switzerland) mixed in 0.02 mL of phosphate buffer saline (PBS). We measured the cutaneous immune response to LPS (Leshchinsky & Klasing, 2001) as the difference in thickness (to the nearest 0.1mm) before and 4.28 ± 0.39 hours after LPS had been injected. This delay corresponds to the peak

of the innate immune response following an injection of LPS in chicken (Parmentier et al., 1998; Leshchinsky & Klasing, 2001). Indeed, 24 hours after LPS injection, the wing web response in chicken was found to be not different to control individuals and did not correlate with wing web response 4 hours after injection (Parmentier et al., 1998). In the second group of nests (n = 40), all nestlings were injected in a similar way with 10 µg of phytohaemagglutinin (PHA, Sigma, L1668, Switzerland) mixed in 0.02 mL of PBS to measure the cutaneous immune response to PHA (Martin et al. 2006b; Smits et al. 1999). We measured this cutaneous response as the difference of wing web thickness before and 25.03 ± 0.18 hours after injection (Smits et al., 1999). In chicken, wing web response to PHA injection after 4 and 24 hours were both significantly different to control individuals and were strongly inter-correlated (Parmentier et al., 1998); the strength of the response was similar at 4 and 24 hours post-injection. We nevertheless measured PHA-response one day post-injection because this is the usual method in the field of immuno-ecology to measure T-cell mediated immunity (Tella et al., 2008). The injected dose of LPS and PHA has been chosen according to previous studies (LPS: Parmentier et al., 1998; PHA: Smits et al., 1999). We obtained a sample of 138 TETRAVAC-nestlings (74 from 45 LPS-nests and 64 from 40 PHA-nests) and 144 control-nestlings (78 from 44 LPS-nests and 66 from 40 PHA-nests).

During this second visit, we also collected two feathers on the back of each nestling to quantify the degree of melanin-based coloration. Briefly, feathers were photographed and pictures imported in the software Adobe Photoshop to measure coloration. We obtained a coloration score by calculating mean hue, saturation and brightness values. As they were highly inter-correlated, we extracted the first component (PC1) of a principal components analysis which explained 78% of the total variance (loading factors for hue, saturation and brightness were 0.61, -0.61 and 0.50, respectively) (for further details see Gasparini et al., 2009 and Piault et al. in press). Low and high PC1 scores stand for dark and pale reddish

melanic coloration, respectively. PC1 values are strongly correlated with colour morphs assigned in the field (r = 0.89) and with reflectance spectra (r = -0.85). Mean sibling coloration did not correlate with brood size (Pearson correlation, r = - 0.03, n = 84, P = 0.82), hatching date (r = -0.10, n = 84, P = 0.34), coloration of foster parents (r = -0.19, n = 76, P = 0.09, foster father coloration was lacking for 8 over 84 nests). Coloration at the nestling stage and at adulthood were highly correlated in 41 individuals (r = 0.71, n = 41, $P < 0.0001$) indicating that coloration measured at the nestling stage is a good surrogate of coloration at adulthood. Within each experimental treatment (TETRAVAC-PHA, TETRAVAC-LPS, PBS-PHA, PBS-LPS) age at which nestlings were injected with TETRAVAC or PBS was not associated with nestling plumage coloration (four mixed model regressions, P-values > 0.10). At each nest visit, we weighed nestlings (to the nearest 1 g) and measured the length of one wing (1 mm) and one tarsus (0.1 mm). These measures were useful to investigate whether mounting a humoral immune challenge affects growth parameters. Nestling blood samples were immediately centrifuged, plasmas placed at -20°C until antibody analyses in autumn 2005, and red blood cells were used for molecular sexing (see Py et al., 2006 for the method). To check whether the vaccine triggered the production of specific antibodies, we measured anti-TETRAVAC antibody concentration in blood plasmas using a sandwich ELISA as described in Gasparini et al. (2009). Optical density (OD) obtained by ELISA provided us with a relative measure of anti-TETRAVAC antibody. The antibody production was estimated as the difference of OD values in blood samples collected ten days apart. For two samples, the amount of collected blood was too low to quantify anti-TETRAVAC antibody concentration, and thus we have a sample of 280 nestlings for which we measured antibody production.

Statistical analyses

We investigated whether the humoral immune challenge (TETRAVAC vs. PBS) affected differentially cutaneous responses to LPS and PHA with mixed model ANCOVAs. The intensity of the response to LPS and PHA was included as a dependent variable in separate models, the treatment (injection of TETRAVAC vs. PBS) as a factor, and nestling coloration, nestling body mass at the second injection (PHA/LPS), brood size and nestling age at the time when TETRAVAC or PBS was injected as three covariates. We controlled for the non-independence of siblings sharing the same nest by incorporating nest identity as a random factor. Statistical analyses were performed using the SAS system (version 9.1; SAS Institute Inc, Cary, NC, USA). Means are quoted ± s.e., statistical tests are two-tailed and *P*-values less than 0.05 are considered significant.

Results

The present study relies on the assumption that our immune treatments did not alter melanin-based coloration in nestlings. Accordingly, nestlings of the four treatments did not differ in plumage coloration (mixed model ANOVA with nest as a random effect: $F_{3,194} = 0.28$, $P = 0.84$).

Vaccinated nestlings produced higher amount of anti-TETRAVAC antibodies (0.076 ± 0.003) than PBS-nestlings (0.008 ± 0.003; mixed model ANOVA with nest as a random categorical variable: $F_{1,195} = 420.14$, $P < 0.0001$). In vaccinated nestlings, anti-TETRAVAC antibody production was not associated with nestling coloration (mixed model ANCOVA with nest as a random categorical variable, coloration: $F_{1,52} = 0.08$, $P = 0.78$) or biological parent coloration (mother: $F_{1,51} = 0.18$, $P = 0.67$; father: $F_{1,46} = 0.05$, $P = 0.82$). We did not detect any effect of the TETRAVAC treatment on nestling tarsus and body mass growth alone or in interaction with nestling coloration (all *P*-values > 0.47).

Cutaneous immune response to LPS was more pronounced in TETRAVAC- than in control-nestlings (Table 1, Figure 1). Mounting a cutaneous response against LPS did not covary with nestling coloration (no effect of coloration alone or in interaction with TETRAVAC treatment, Table 1). Conversely, mounting a cutaneous response to PHA was significantly explained by the interaction between nestling coloration and TETRAVAC treatment (Table 1). When injected with TETRAVAC dark reddish melanic nestlings mounted a stronger cutaneous response to PHA than pale reddish ones (similar mixed model ANCOVA as in Table 1, $F_{1,21} = 5.78$, $P = 0.03$, $\beta \pm se = -0.066 \pm 0.027$; Figure 2), a relationship that was not detected in PBS-nestlings ($F_{1,24} = 0.90$, $P = 0.35$**,** $\beta \pm se = 0.025 \pm 0.027$). Similar statistical models where coloration of biological or foster parents were entered as a covariate in place of nestling coloration showed that cutaneous responses to LPS and PHA were neither significantly correlated with coloration of biological and foster parents alone nor in interaction with the TETRAVAC treatment (biological parents: $P > 0.06$; foster parents: $P > 0.08$).

Discussion

Most studies that have investigated the synergy between immune components have shown that investment in a second immune response is penalized by investment in a previous immune challenge (e.g. Goüy de Bellocq et al., 2006; Martin et al., 2006a; Forsman et al., 2008 but see also Lindström et al., 2004). In contrast, our results suggest that a humoral immune challenge activates the cutaneous immune response to LPS (Figure 1). Cutaneous response to LPS mainly involves the innate immune response and its enhancement following a humoral immune challenge appears to be adopted by all nestlings independently of their coloration. Therefore, melanogenesis is not associated with the synergistic interaction between the humoral challenge and innate immunity. To our knowledge, this result provides

the first example in a natural population of the synergistic action between humoral and innate immunity, a well known physiological mechanism coming from immunological studies (Janeway et al., 2005). When considering the cutaneous response to PHA, this synergistic action only prevailed in dark reddish melanic individuals for whom the cutaneous response to PHA was higher in TETRAVAC- than control-nestlings (Figure 2). In contrast, pale reddish melanic individuals showed reduced intensity of the cutaneous immune response to PHA after a first humoral immune challenge. Cutaneous response to PHA mainly involves the T-cell part of the immune system (Tella et al., 2008) and in our owl population the degree of melanin-based coloration is associated with allocation towards this component of the immune system, dark and pale reddish melanic individuals increasing and decreasing, respectively, the intensity of T-cell immune response after a humoral immune challenge. Because we cross-fostered eggs to allocate genotypes randomly among environments (as shown by the absence of correlation between coloration of foster and biological parents), we suggest that the link between melanin-based coloration and the allocation of resources in cutaneous response to PHA following a humoral immune challenge is due to pre-hatching maternal or genetic factors. We temptingly exclude that this link is due to a maternal transfer of egg components (pre-hatching maternal factor) associated with melanin-based coloration because we did not find any significant interaction between parental coloration and pre-immune challenge on cutaneous immune response to PHA. Our results are rather consistent with linkage disequilibrium between melanin-based coloration and energy reallocation towards the T-cell mediated immunity after a humoral immune challenge.

Such association is interesting in the light of the field study performed by Galeotti & Sacchi (2003) who reported that in Italian tawny owls the level of blood parasites increased with the degree of melanic reddishness. Two alternative scenarios may explain this relationship. First, reddish owls have a weaker immune system and thereby they become more

intensely infected by parasites. Alternatively, reddish owls exploit environments where parasites are more abundant, and as a consequence these owls have evolved a potent immune system. In agreement with this alternative scenario, a recent study showed that after an immune challenge dark reddish breeding females mount a stronger and prolonged humoral immune response vaccination at the cost of body mass maintenance when compared to pale reddish conspecifics (Gasparini et al., 2009). This suggests that the degree of reddishness is associated with adaptation to resist parasite attacks. Assuming that dark reddish individuals exploit environments where parasites are particularly abundant or virulent, they may invest more resources in their immune system, and thus a first infection may trigger defence mechanisms in prevision of subsequent parasite attacks. In contrast, when individuals are only occasionally exposed to parasites, as it might be the case for pale reddish individuals, down-regulation of the immune system may be adaptive to save energy for other demanding activities such as growth particularly when food resources are limited (Piault et al., in press). Assuming that parasite exposure varies in space and time even at small scale (May & Southwood, 1990), both strategies may coexist within the same population. Altogether results of the present study and of Gasparini et al. (2009) and Galeotti & Sacchi (2003) are consistent with the hypothesis that the degree of melanin-based coloration is positively associated with investment in the immune system at the expense of body maintenance. Studies where differently coloured owls are experimentally exposed to parasites are required to validate this scenario.

In conclusion, our study suggests that mounting a humoral immune response can stimulate another component of the immune system in all individuals or only in a subset of genotypes as revealed by melanin-based coloration, a trait for which the expression is under strong genetic control in the tawny owl (Gasparini et al. 2009). Synergistic and antagonistic interactions between branches of the immune system can take place in a natural population

depending on immune branches (synergistic interaction for humoral and innate immunity) and individual genotypes (synergistic interaction in dark reddish melanic nestlings for humoral and T-cell immunity; antagonistic interaction in pale reddish melanic nestlings for humoral and T-cell immunity). In addition, these results re-enforce the recent hypothesis proposing that melanin-based coloration is associated with a continuum of resource allocation strategy in key fitness components including immunity.

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Table 1: Mixed model ANCOVAs testing whether a humoral immune challenge against TETRAVAC affects non-specific (LPS) and T-cell immune response (PHA) in interaction with nestling melanin-based coloration. Humoral immunisation (TETRAVAC or PBS) was a factor, and nestling coloration, age and mass at the time when TETRAVAC or PBS was injected three covariates. The nest identity was included as a random factor to avoid pseudoreplication. Among nestlings injected with LPS, nestling coloration and the interaction between treatment and coloration were not significant ($P = 0.68$ and $P = 0.67$, respectively) and therefore removed from the final model. Similarly, brood size and nestling age at TETRAVAC or PBS injection were not significant and were removed from final models ($P > 0.15$ and $P > 0.06$, respectively). *F*-values are given for fixed effects and Wald *Z*-values for random effects.

Variable	**Non-specific immune response (LPS)**			**T-cell immune response (PHA)**		
Source	*F* or Wald *Z*	d.f.	*P*	*F* or Wald *Z*	d.f.	*P*
Fixed effects						
Nestling mass at LPS/PHA injection	7.86	1,106	0.006	5.90	1,86	0.02
Treatment (TETRAVAC/PBS)	4.83	1,106	0.03	0.25	1,86	0.62
Nestling coloration	–	–	–	0.70	1,86	0.41
Treatment x nestling coloration	–	–	–	4.83	1,86	0.03
Random effects						
Nest identity	2.18		0.01	2.66		0.004

Figure 1: Mean ± s.e. of the response to LPS (mm) in nestling tawny owls previously injected either with the TERAVAC vaccine or PBS.

Figure 2: Relationship between residuals of cutaneous immune response to PHA and degree of melanin-based coloration (PC1) in nestling tawny owls. Residuals were extracted from the regression of response to PHA on nestling body mass.

Figure 1

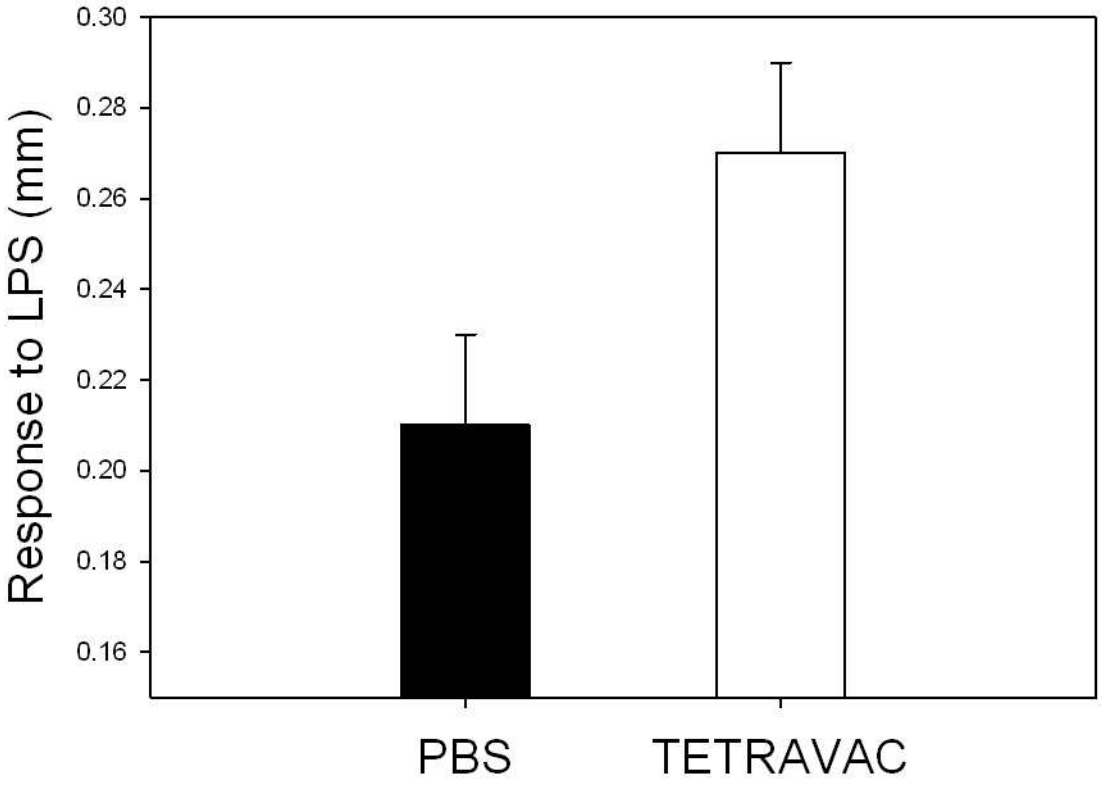
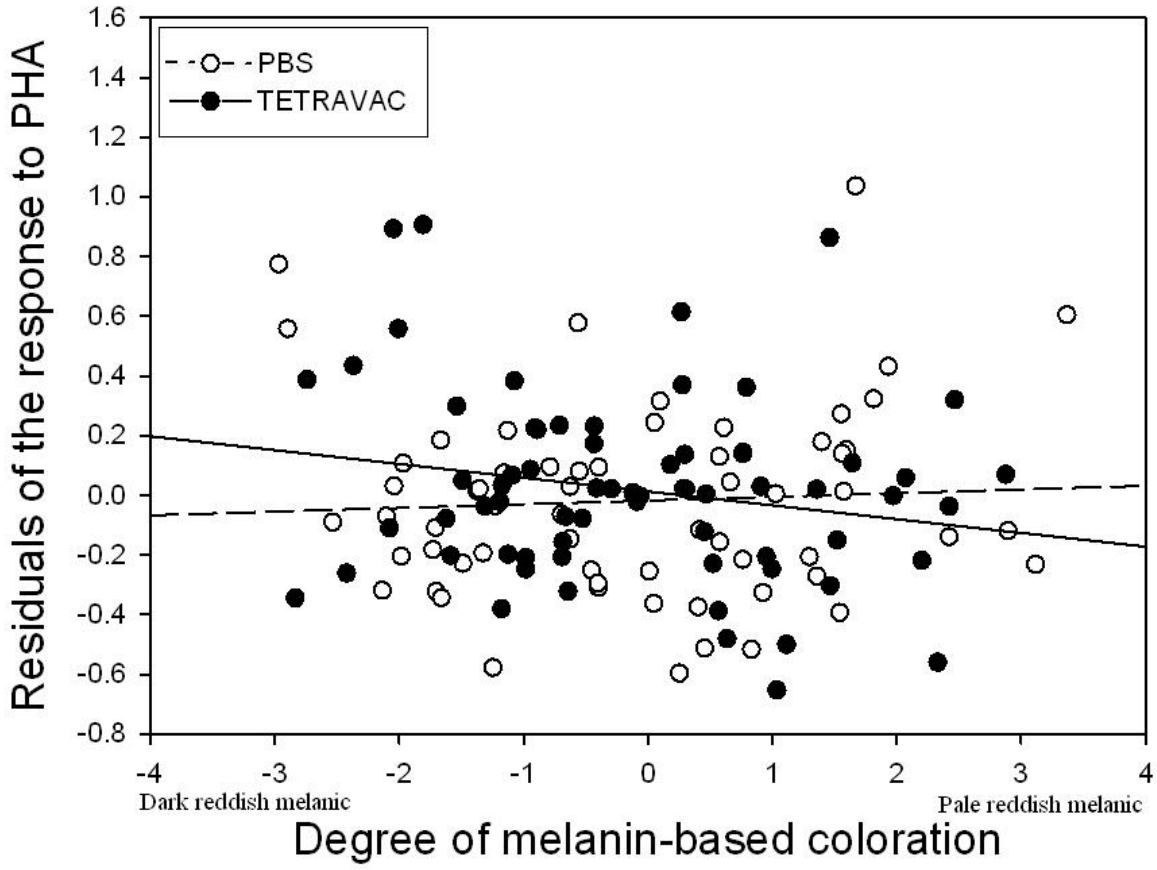

0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
Response to LPS (mm)
PBS
TETRAVAC

Figure 2


PBS
TETRAVAC
Residuals of the response to PHA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-4
-3
-2
-1
0
1
2
3
4
Dark reddish melanic
Pale reddish melanic
Degree of melanin-based coloration