Title: Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*

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Running title: Melanism, immunity and life-history trade-offs in *S. littoralis*

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Abstract

Several insect species show an increase in cuticular melanism in response to high densities. In some species, there is evidence that this melanism is correlated with an up-regulation of certain immune system components, particularly phenoloxidase (PO) activity, and with the down-regulation of lysozyme activity, suggesting a trade-off between the two traits. As melanism has a genetic component, we selected both melanic and non-melanic lines of the phase-polyphenic lepidopteran, *Spodoptera littoralis*, in order to test for a causative genetic link between melanism, PO activity and lysozyme activity, and to establish if there are any life-history costs associated with the melanic response. We found that, in fact, melanic lines had lower PO activity and higher lysozyme activity than non-melanic lines, confirming a genetic trade-off between the two immune responses, but also indicating a genetic trade-off between melanism and PO activity. In addition, we found that lines with high PO activity had slower development rates and lower pupal weights, suggesting that investment in PO, rather than melanism, is costly.

Introduction

The use of colour is ubiquitous in the animal kingdom, with pigments such as melanin being employed for a variety of purposes, including camouflage (Kettlewell 1973; Majerus 1998; Hoekstra & Nachman 2003) warning colouration, usually in conjunction with contrasting colours such as reds or yellows (Wiklund & Sillen-Tullberg 1985; Marples *et al.* 1994; Kauppinen & Mappes 2003; Bezzerides *et al.* 2007); and in sexually-selected traits (Jarvi & Bakken 1984; Moller 1988; Siva-Jothy 2000; Rosen & Tarvin 2006). In many cases, these pigments appear to be used as signals of health or quality, the honesty of which can be maintained only if the signal is costly to produce (Zahavi 1975; Sheldon & Verhulst 1996). Melanin and its precursors also play a protective role against parasites in both vertebrates and invertebrates (Söderhall & Ajaxon 1982; Montefiori & Zhou 1991; Nappi & Vass 1993; Marmaras *et al.* 1996; Mackintosh 2001; Griffith *et al.* 2006), potentially creating a direct link between the display and parasite resistance (e.g. Kose & Møller 1999).

In insects, melanin and its precursors are used directly in the immune system. Phenoloxidase (PO), a key enzyme in the synthesis of melanin, is found in the haemolymph, midgut and cuticle. It is thought to be involved in non-self recognition, as well as the encapsulation of larger organisms, and so plays a crucial role in the insect immune response (Ashida & Brey 1995; Ashida & Brey 1997; Wilson *et al.* 2001; Cotter *et al.* 2004a). Melanin itself also has chemical properties that may inhibit fungal growth (Söderhall & Ajaxon 1982; St. Leger *et al.* 1988).

In a number of insect species, melanin is deposited in the cuticle in response to increasing population density, resulting in density-specific morphs or phases. The archetypal density-

dependent phase polyphenic species, the desert locust, *Schistocerca gregaria*, undergoes a number of morphological, physiological and behavioural changes in response to increasing population density, one of which is the melanisation of the cuticle. However, this phenomenon also occurs in a number of orthopteran, lepidopteran and phasmid species (Wilson & Cotter 2008 and references therein). The adaptive function of melanism in the high density phase has not been categorically established, but there is strong evidence to suggest that it is associated with increased investment in the immune system. The density-dependent prophylaxis (DDP) hypothesis posits that as many parasites are transmitted in a positively density-dependent fashion, and investment in the immune system is assumed to be costly, it would be beneficial for individuals to use the density of conspecifics as a cue to the risk of parasitism and to tailor investment in immune function accordingly (Wilson & Reeson 1998).

Previous studies on several phase polyphenic lepidopteran species have found that the high-density or melanic phase is more resistant to viruses (Kunimi & Yamada 1990; Goulson & Cory 1995; Reeson *et al.* 1998), entomopathogenic fungi (Mitsui & Kunimi 1988; Wilson *et al.* 2001) and parasitoids (Wilson *et al.* 2001). Similarly, both melanic mealworm beetles (Barnes & Siva-Jothy 2000) and high-density desert locusts (Wilson *et al.* 2002) were found to be more resistant to an entomopathogenic fungus than their non-melanic or low-density counterparts.

The changes that occur in the immune system that underlie this change in susceptibility to parasites are less clear, but there is evidence that in high-density phenotypes there is an increase in either PO activity (Reeson *et al.* 1998; Wilson *et al.* 2001; Cotter *et al.* 2004a), encapsulation ability (Cotter *et al.* 2004a), haemocyte density (Wilson *et al.* 2002) and/or

lysozyme-like antibacterial activity (Wilson *et al.* 2002). However, in most cases, not all immune traits are simultaneously up-regulated and, indeed, there is evidence from the phase polyphenic lepidopteran *Spodoptera littoralis*, for a trade-off between PO activity and lysozyme-like antibacterial activity (Cotter *et al.* 2004a; Cotter *et al.* 2004b), suggesting that all immune function traits cannot be simultaneously upregulated.

Despite the wealth of evidence that melanism is associated with increased immune function in species that show a melanic response to high densities, it is still unclear if this relationship is simply correlational or if there is a causative link. To test this, we selected for both melanism and non-melanism (paleness) in the phase polyphenic lepidopteran, *Spodoptera littoralis* (the Egyptian cotton leaf worm). We have shown previously that although melanism is triggered by high densities, it also has a strong additive genetic component, with some families becoming melanic at low densities and others remaining pale at high densities (Cotter *et al.* 2004b; Lee & Wilson 2006). We then asked the following questions:

1. Does selection for melanism result in changes in immune system traits, such as PO activity and lysozyme-like antibacterial activity?
2. Is there evidence for a trade-off between PO activity and lysozyme-like antibacterial activity within or across selected lines?
3. Are there life-history costs associated with selection for melanism?

From our previous finding that high density, melanic individuals had high PO activity but low lysozyme activity, we predicted that if melanism was directly linked to the immune system, then melanic selected lines would show increased PO activity and decreased antibacterial activity, with pale lines showing the reverse trend. Following this, we also

predicted that, in the absence of parasitism, melanic lines would show a fitness cost compared to pale lines, as investment in immunity should be costly.

Methods

Spodoptera littoralis culture

The *Spodoptera littoralis* culture was established from eggs collected near Alexandria in Egypt in 2002, and high numbers were maintained at each generation to reduce inbreeding. At the start of the selection experiment, the colony had been reared using single pair mating for 18 generations with over 150 pairs established each generation. Larvae were reared singly from the 2nd instar on a semi-artificial wheatgerm diet in 25 ml polypots (Cotter 2002).

Selection regime

At the beginning of the selection experiment, two replicate groups of 200 larvae were selected from the colony, placed in individual polypots and reared in one of two incubators under a 12:12 light:dark regime at 25°C until the final instar. In the middle of the final instar, just prior to the wandering phase (where larvae cease feeding and start to look for a place to pupate) larvae were numbered, weighed and colour-scored by eye, which involved placing them into one of seven categories: extra pale, pale, pale intermediate, intermediate, dark intermediate, dark and extra dark (inset, Figure 1). For each replicate, the 100 darkest larvae were assigned to the dark line (1D – dark line, replicate 1, 2D – dark line replicate 2) and the 100 palest larvae were assigned to the pale line (1P – pale line, replicate 1, 2P – pale line

replicate 2). Emerging moths were mated in groups of 10 males and 10 females in breeding chambers with access to sucrose solution and tissue paper for egg laying. Approximately 100 adults in each replicate were allowed to breed each generation.

Generations 1 – 4: 400 larvae were set up per line each generation, larvae were colour scored by eye in the final instar and the 25% darkest (1D and 2D) or 25% palest (1P and 2P) were selected to breed. Generations 5 – 11: 600 larvae were set up per line each generation, larvae were colour-scored by eye in the final instar and the 20% darkest (1D and 2D) or 20% palest (1P and 2P) were selected to breed. At generation 12, selection was relaxed but as the colour of the larvae began to slip back towards that of larvae in the control line, a 50% selection pressure was re-instituted at generation 14 and maintained thereafter.

In generations 0, 7, 11 and 12, larvae were additionally colour-scored using an Avaspec-2048 fibre optic spectrometer with an AvaLight-HAL tungsten halogen light source (Avantes, Eerbeek, The Netherlands). Measurements were taken using a 2-mm diameter bifurcated fibre optic probe that was positioned at a 90° angle to the integument surface of each insect (Lee & Wilson 2006). A cylindrical plastic tube was attached to the probe in order to maintain a constant distance of 2 mm from the sample. A late final-instar *S. littoralis* caterpillar with extremely conspicuous pale colouration was used to set the white standard reference, while the dark standard was established by eliminating light from the probe. These standards allowed the quantification of the relative paleness of a sample compared with the white standard reference, which was expressed as an absorbance value (%). Thus, 0% absorbance was equivalent paleness to the white standard, while 100% absorbance was equivalent to the dark standard. Triplicate absorbance values were recorded at 575 nm

wavelength for each larva along the dorsal midline of the cuticle. The repeatability of this technique was high ($r = 0.86 +/- 0.009$, $n = 530$, 3 measurements per individual).

The correlation between the qualitative categorical colour score and the quantitative spectrometer score was found to be extremely strong ($r = 0.81$, $t_{503} = 30.93$, $P < 0.001$, Fig. 1), therefore the qualitative scores were changed to the mean spectrometer score for that category to allow the response to selection to be quantified more easily.

Haemolymph sampling

All immune function data were collected from larvae during generation 12; 100 larvae were sampled for each line. After colour-scoring, larvae were weighed and a haemolymph sample was taken from each individual by piercing the final proleg with a fine needle and collecting the haemolymph in an Eppendorf tube. All of the samples were then frozen at -80°C until they were to be measured. After the haemolymph was sampled, larvae were returned to their polypots to pupate.

Phenoloxidase assay

Haemolymph PO was measured using a modified version of the method described in Cotter & Wilson (2002). In brief, 8 μl of haemolymph were added to 400 μl of ice-cold phosphate buffered saline (pH 7.4) in a plastic Eppendorf tube and vortexed. PO activity was assayed spectrophotometrically with dopamine as a substrate. This assay involved adding 100 μl of 4

mM dopamine to 100 µl of the buffered haemolymph and incubating duplicate samples of the mixture on a temperature-controlled *VERSAmax* tuneable microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at 25°C. PO activity was expressed as the change in absorbance over the first 10 minutes, which is during the linear phase of the reaction.

Protein assay

Protein was measured using the *BioRad* protein assay kit with BSA as the protein standard. Two replicates of 5 µl of the haemolymph/PBS mixtures were used to measure the protein in each sample. Absorption was measured on a temperature-controlled *VERSAmax* tuneable microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at 600 nm.

Lysozyme-like antibacterial activity

Lytic activity against the bacterium, *Micrococcus lysodeikticus* (Sigma) was determined using a lytic zone assay. Agar plates containing 12 ml of 1 % agar with 5 mg per ml freeze-dried *M. lysodeikticus* were prepared as described in Kurtz et al (2000). For each plate, 20 holes with a diameter of 2 mm were punched in the agar and 1 µl of haemolymph was placed in each well, two replicates per sample. The plates were incubated at 33ºC for 18 hours then photographed using a *Polaroid DMC* digital camera and the diameter of the clear zones calculated using *Image Pro Plus* software (Media Cybernetics, Silver spring, MD, USA).

Standard curves were obtained using a serial dilution of hen egg white lysozyme (BDF). Concentration of "hen egg white lysozyme equivalents" was then calculated.

Life history traits

Life history data were collected from larvae during generations 12 and 16. In generation 12, pupation date, pupal weight, emergence date and sex were recorded for each individual. However, these individuals had been wounded and had lost blood during haemolymph sampling which may have affected subsequent life history measurements. Therefore, the same life-history data were collected from non-sampled individuals during generation 16.

Statistical analyses

The realized heritability for cuticular melanism was calculated for each line by plotting the mean colour score for each generation against the cumulative selection differential. The expected selection differential was calculated as the deviation of the mean cuticular colour of the selected individuals in each generation from the population mean before selection. This was then summed each generation to give the cumulative selection differential. The realised heritability (h^2) was then calculated from the slope of the regression of mean colour score (R) against the cumulative selection differential (S), as $h^2 = R/S$ (Falconer & Mackay 1996).

The effects of selection on the immune function traits, haemolymph protein levels, larval weight and life history traits were analysed using REML mixed models in Genstat 10. Selection experiment data are typically analysed either by comparing line means (e.g.

Armitage & Siva-Jothy 2005; Schwarzenbach & Ward 2006), or by using an ANOVA/ REML approach including selection lines and replicates as factors in the model (eg (Evans *et al.* 2006; Vermeulen & Bijlsma 2006; McKean & Nunney 2008). Whilst both approaches are valid, we chose to use a REML-based analysis so that we could look at additional sex and condition effects on immune function and life history traits. Replicate was included as a random effect with Line, Sex and Line-nested-within-Replicate included as fixed effects. For the immune function and protein traits, two sets of models were examined, either with Weight included as a covariate or without to account for the effects of condition on immune function investment. For all models, if there was a significant effect of Line nested within Replicate, data were analysed for each replicate separately. To examine correlations between the measured traits, Pearson's correlation coefficients were calculated using S-Plus 7.

Results

Response to selection and realised heritability of cuticular melanism

For the first four generations of selection, the response in cuticular colour was minimal. The two pale lines did seem to get steadily paler, albeit at a very slow rate, but the dark lines showed no obvious response to selection (Fig. 2a). After generation 5, the selection differential was increased from 25% to 20%, which seemed to result in a much greater response in all four of the selected lines (Fig. 2a). Relaxation of selection at generation 12 resulted in the mean colour of all lines starting to slip back towards the controls, suggesting that both paleness and darkness are in some way costly to maintain.

The regression of cumulative selection differential on response was highly significant for each of the lines (**1D**, slope ± SE = 0.14 ± 0.02, $F_{1,11}$ = 33.97, P <0.001; **2D**, slope ± SE = 0.22 ± 0.03, $F_{1,11}$ = 51.69, P <0.001; **1P**, slope ± SE = 0.21 ± 0.03, $F_{1,11}$ = 51.44, P <0.001; **2P**, slope ± SE = 0.20 ± 0.03, $F_{1,11}$ = 35.71, P <0.001; Fig. 2b) giving heritability estimates (h^2 ± SE) for cuticular melanism in each of the lines as 0.14 ± 0.02 (**1D**), 0.22 ± 0.03 (**2D**), 0.21 ± 0.03 (**1P**) and 0.20 ± 0.03 (**2P**).

Effect of selection on condition and immune function traits

Larval weight: Selection for cuticular melanism had a significant effect on larval weight. There was a significant replicate by line interaction (Wald statistic χ^2_2 = 8.10, P = 0.019); when analysed separately, dark lines were heavier than pale lines in replicate 1 only (replicate 1: Wald statistic χ^2_1 = 9.18, P = 0.003; replicate 2: Wald statistic χ^2_1 = 0.34, P = 0.562, Fig. 3a).

Haemolymph protein levels: Selection for cuticular melanism had a significant effect on protein levels in the haemolymph. Dark lines had significantly higher haemolymph protein levels than pale lines (Wald statistic χ^2_1 = 14.37, P < 0.001, Fig 3b). There was no interaction between replicate and line and so the term was removed from the model (Wald statistic χ^2_2 = 0.62, P = 0.734). There was a significant positive effect of larval weight on protein levels (Wald statistic χ^2_1 = 26.61, P < 0.001), but again the inclusion of larval weight in the model did not change the trends but the difference between the lines was reduced (Wald statistic χ^2_1 = 11.31, P < 0.001).

Female larvae had significantly higher protein levels than male larvae, both with the inclusion of weight in the model (Wald statistic $\chi^2_1 = 16.92$, $P < 0.001$) and without it (Wald statistic $\chi^2_1 = 21.24$, $P < 0.001$).

Phenoloxidase activity: Selection for cuticular melanism had a significant effect on PO activity in the haemolymph. Pale lines had significantly higher PO activity than dark lines (Wald statistic $\chi^2_1 = 41.92$, $P < 0.001$). Although, there was a significant replicate by line interaction (Wald statistic $\chi^2_2 = 15.04$, $P < 0.001$), when analysed separately, pale lines had higher PO activity than dark lines in both replicates, but the effect was much more pronounced in replicate 2 (replicate 1: Wald statistic $\chi^2_1 = 3.92$, $P = 0.047$; replicate 2: Wald statistic $\chi^2_1 = 49.11$, $P < 0.001$, Fig. 3c)

There was a significant negative effect of larval weight on PO activity (Wald statistic $\chi^2_1 = 13.23$, $P < 0.001$). The inclusion of larval weight in the model did not change the trends but the difference between the lines was reduced. The interaction between replicate and line was still significant (Wald statistic $\chi^2_2 = 19.72$, $P < 0.001$). In replicate 2, pale larvae had higher PO activity than dark larvae (Wald statistic $\chi^2_1 = 51.19$, $P < 0.001$), but in replicate 1 the inclusion of weight in the model made the difference between the lines non-significant (Wald statistic $\chi^2_1 = 1.41$, $P = 0.237$).

Female larvae had significantly higher PO activity than male larvae both with the inclusion of weight in the model (Wald statistic $\chi^2_1 = 7.33$, $P = 0.007$), and without it (Wald statistic $\chi^2_1 = 4.74$, $P = 0.03$).

Lysozyme activity: Selection for cuticular melanism also had a significant effect on lysozyme-like antibacterial activity in the haemolymph. There was no interaction between replicate and line and so the term was removed from the model (Wald statistic $\chi^2_2 = 4.36$, P = 0.113). Dark lines had significantly higher lysozyme activity than pale lines (Wald statistic $\chi^2_1 = 7.89$, P = 0.005, Fig 3d). There was also a significant positive effect of larval weight on lysozyme activity (Wald statistic $\chi^2_1 = 6.52$, P = 0.011). As for PO activity, the inclusion of larval weight in the model did not change the trends but the difference between the lines was reduced (Wald statistic $\chi^2_1 = 6.67$, P = 0.01).

Female larvae had significantly higher lysozyme activity than male larvae, both with the inclusion of weight in the model (Wald statistic $\chi^2_1 = 5.60$, P = 0.018) and without it (Wald statistic $\chi^2_1 = 6.68$, P = 0.01).

Effect of selection on life history traits

Time to pupation: Selection for cuticular melanism had a significant effect on the time spent in the larval stage from egg hatching to pupation (Wald statistic $\chi^2_1 = 117.66$, P < 0.001). The interaction between replicate and line was significant (Wald statistic $\chi^2_2 = 26.77$, P < 0.001), but in both replicates pale larvae took longer to pupate than dark larvae (replicate 1: Wald statistic $\chi^2_1 = 114.84$, P < 0.001; replicate 2: Wald statistic $\chi^2_1 = 17.37$, P < 0.001, Fig. 4a). Sex was also significant, with females taking slightly longer to pupate than males (females 18.65 $\pm$ 0.12, males 18.10 $\pm$ 0.12 days; Wald statistic $\chi^2_1 = 21.33$, P < 0.001).

Pupal weight: Selection for cuticular melanism had a significant effect on pupal weight (Wald statistic $\chi^2_1 = 18.98$, $P < 0.001$). However, the interaction between replicate and line was again significant (Wald statistic $\chi^2_2 = 27.06$, $P < 0.001$), with the pale line pupating at a lower weight than the dark line in replicate 2 only (replicate 1: Wald statistic $\chi^2_1 = 0.15$, $P = 0.703$; replicate 2: Wald statistic $\chi^2_1 = 52.93$, $P < 0.001$, Fig. 4b). Sex was also significant, with females pupating at a heavier weight than males (females, 353.6 $\pm$ 2.6 mg, males, 319.2 $\pm$ 2.6 mg, Wald statistic $\chi^2_1 = 181.59$, $P < 0.001$).

Growth rate: The two measures above can be combined as growth rate in mg gained per day of feeding (i.e. pupal weight divided by time to pupation). Growth rate was significantly higher in the dark lines (Wald statistic $\chi^2_1 = 98.26$, $P < 0.001$; Fig. 4c); the interaction between line and replicate was not significant (Wald statistic $\chi^2_2 = 3.59$, $P < 0.167$). Sex was significant, with females growing at a faster rate than males (females 19.4 $\pm$ 0.2, males 18.0 $\pm$ 0.2 mg per day, Wald statistic $\chi^2_1 = 65.98$, $P < 0.001$).

Time to emergence: Selection for cuticular melanism also had a significant effect on the time spent in the pupal stage (Wald statistic $\chi^2_1 = 177.55$, $P < 0.001$). The interaction between replicate and line was significant (Wald statistic $\chi^2_2 = 822.7$, $P < 0.001$); in both replicates, pale larvae took longer to emerge as adults than dark larvae, though the effect was marginally non-significant in replicate 2 (replicate 1: Wald statistic $\chi^2_1 = 265.06$, $P < 0.001$; replicate 2: Wald statistic $\chi^2_1 = 2.91$, $P = 0.08$, Fig. 4d). Sex was also significant, with females emerging

more than a day earlier than males (females: 11.13 ± 0.05; males: 12.51 ± 0.05 days; Wald statistic $\chi^2_1 = 726.01$, $P < 0.001$).

Relationship between cuticular melanism and immunity within and across lines

Melanism and haemolymph PO: Previous work on this species has shown positive correlations between haemolymph PO activity and cuticular melanism, which led us to predict that selection for melanism would result in increased levels of haemolymph PO activity. As we have shown the opposite result here, we examined the relationship both within and across lines to understand better the effects of selection on these traits. Using a mixed-model analysis, as before but including melanism (i.e. larval cuticular absorbance) as a covariate in the model, there was again a significant interaction between replicate and line (Wald statistic $\chi^2_2 = 15.75$, $P < 0.001$). However, in both replicates, with line included in the model, there was a significant positive effect of larval melanism on PO activity (replicate 1: Wald statistic $\chi^2_1 = 3.77$, $P = 0.05$; replicate 2: Wald statistic $\chi^2_1 = 6.18$, $P = 0.014$), whilst across all data the relationship was negative ($r = -0.31$, $t_{303} = -5.56$, $P < 0.001$; Fig. 5a).

Melanism and lysozyme: Although the overall correlation between melanism and lysozyme-like antibacterial activity was positive, ($r = 0.18$, $t_{282} = 3.08$, $P = 0.002$; Fig. 5b), there was no significant relationship between melanism and lysozyme activity within lines (Wald statistic $\chi^2_1 = 0.27$, $P = 0.606$).

Discussion

Selection for both increased and decreased levels of cuticular melanism was successful, with significant divergence between the lines occurring by the fifth generation. The realised heritability estimates ranged from 0.14 to 0.22, which is slightly lower than previous estimates obtained for this species using sib-analysis (0.36 $\pm$ 0.08; Cotter *et al.* 2004b) and parent-offspring regression (0.18 - 0.30; Lee & Wilson 2006). Nonetheless, it confirms the finding that variation in melanism in this species has both additive genetic and environmental components. We then considered whether melanism was directly related to immune function by examining immune traits in the selected lines. We found that there was a causative relationship between melanism and immunity, as selection for both paleness and darkness resulted in a correlated response to selection in both PO activity and lysozyme-like antibacterial activity. In addition, we found that there were life-history costs associated with melanism (and investment in PO activity), as decreased melanism (and high PO activity) were correlated with a slower development time, lower growth rate and slower time to adult emergence.

Due to the physiological relationship between PO activity and melanin (Cotter *et al.* 2004a), we predicted that selection for melanism would result in increased haemolymph PO activity. Contrary to our prediction, we found that haemolymph PO activity was higher in the pale-selected lines. As before, we found that the correlation between PO activity and melanism was positive *within* lines, but *across* lines the correlation was negative. This pattern is typical of a situation where variation in resource acquisition is greater than variation in resource allocation (van Noordwijk & de Jong 1986), and so generates positive phenotypic correlations between traits; individuals with higher levels of resources can afford to invest in both haemolymph PO and cuticular melanism, whereas those with fewer resources have

lower levels of both. Only when we consider the relationship across selected lines is the trade-off between the two traits (melanism and PO activity) revealed.

The mechanism driving this trade-off is unclear, however one possibility is a trade-off between the manufacture of granular and haemolymph PO. Granular PO, which is biochemically distinct from haemolymph PO, is synthesised in the epidermis and transported to the cuticle, and has been shown to be responsible for cuticular melanisation in the tobacco hornworm, *Manduca sexta* (Hiruma & Riddiford 1988). It is possible that the requirement for large amounts of granular PO in the dark-selected lines results in a shortage of the necessary amino acids or copper required for the manufacture of haemolymph PO. A previous study with mealworm beetles found that selection for melanism resulted in increased haemolymph PO activity, suggesting that the mechanisms controlling cuticular melanism may be different in the two species (Armitage & Siva-Jothy 2005).

We also predicted that pale lines would have increased lysozyme activity due to the negative genetic and phenotypic correlations previously found between lysozyme and PO activity in this species (Cotter *et al.* 2004a; Cotter *et al.* 2004b). In fact, lysozyme activity was higher in the dark lines. However, this pattern of PO activity levels being higher and lysozyme activity levels being lower in the pale lines than in the dark lines, concurs with our previous finding of a genetic trade-off between these two traits (Cotter *et al.* 2004b).

Protein levels in the haemolymph could be considered as a measure of condition (Cotter *et al.* 2004a; Cotter *et al.* 2008), and have also been shown to be correlated with resistance to bacterial infection in crickets (Adamo 2004). Haemolymph protein levels followed the same pattern as lysozyme activity, being higher in the dark than the pale selected lines. This suggests that dark larvae are in better condition than pale larvae, and that, in conjunction with

the lysozyme levels, they would be better able to resist bacterial infection. However, condition alone cannot account for the differences in immune function, as inclusion of weight in the models had no effect on the observed patterns, with the exception of PO levels in replicate 1, where the difference between the lines became marginally non-significant.

Selection for cuticular melanism also revealed life-history trade-offs, with pale larvae taking longer to pupate, having a slower growth rate and taking longer to emerge as adults than dark larvae. Larval and pupal weight were lower in pale larvae in one replicate only, it is possible therefore that this difference was simply due to drift. Our prediction was that dark lines would pay this life-history cost, as we assumed that it would be that dark larvae that invested more heavily in haemolymph PO activity. However, the results do confirm the prediction that investment in haemolymph PO activity carries life-history costs. There are several possibilities for the nature of these costs; as PO is manufactured in haemocytes, investment in PO activity might require the production of additional haemocytes, which would require additional resources. Another possibility is that, due the cytotoxic nature of the intermediates produced during PO activation, it is necessary to store PO in its inactive form, proPO, which is maintained by proteinase inhibitors (Nappi & Vass 1993; Cerenius & Soderhall 2004). Investment in high levels of both PO and these proteinase inhibitors could be costly in terms of protein resources, which would otherwise be used for growth. It is worth noting that without the PO data, it may appear that selection for and against melanism had simply resulted in high- and low-quality lines respectively; i.e., that melanic larvae were equivalent to Spitze's "superfleas" (Spitze 1991; Reznick *et al.* 2000). Our results therefore emphasise the importance of measuring multiple traits when looking for fitness costs (Reznick *et al.* 2000).

In addition, it is worth noting that whilst larvae weren't subject to predation in the lab, melanic lines may experience predation costs in the field. Armyworms typically feed on green foliage and highly melanic individuals would suffer increased conspicuousness against this background. Previous studies have shown that conspicuous melanic colouration can increase the risk of predation in the field (but see Wilson 2000). For example, Svensson & Friberg (Svensson & Friberg 2007) found evidence for selection on melanic wing patch colour and size in *Calopteryx* spp. that had been subject to predation by birds, and melanic *Daphnia* morphs of were shown to be subject to greater predation by trout than transparent morphs (Saegrov *et al.* 1996). Increased activity levels previously reported in melanic larvae may also increase their risk of predation (Hodjat, 1970). Furthermore, increased activity and melanism are both associated with increased dopamine levels in *Drosophila*, the metabolism of which is a major source of reactive oxygen species, thought to contribute to early senescence (Vermeulen & Bijlsma 2006). Indeed, a recent study in yellow dung flies found that lines selected for high PO levels showed reduced longevity under starvation conditions, possibly due to a concomitant increase in the PO substrate dopamine ((Schwarzenbach & Ward 2006). A further examination of these potential costs is an interesting area for future research.

An interesting additional finding was that, relative to males, females had higher levels of both PO and lysozyme activity, higher protein levels, faster growth rate and heavier pupal weight. This mirrors results from other insect species showing that females tend to invest more heavily in the immune system than males, possibly due to higher reproductive success being attained through longevity in females than males ((Rolff 2002), and references therein).

In conclusion, it appears that larval melanism is causally linked to immune function investment in this species but, contrary to our expectation, that PO in the haemolymph is traded-off against melanin in the cuticle, though the mechanism behind this trade-off is currently unknown. Selection for melanism confirms the trade-off within the immune system previously reported from this species (Cotter *et al.* 2004a; Cotter *et al.* 2004b), and also identified in other insect species based on negative phenotypic correlations between PO and lysozyme activity (Moret & Schmid-Hempel 2001; Moret & Siva-Jothy 2003; Rantala & Kortet 2003). Indeed, a recent study using *Trichoplusia ni* larvae found that the inclusion of non-pathogenic bacteria in the diet resulted in the up-regulation of lysozyme activity, but a down-regulation of PO activity (Freitak *et al.* 2007). Furthermore, examination of gene expression in the midguts of these larvae found that several antibacterial genes were up-regulated, including lysozyme, but that PO inhibiting enzyme was also up-regulated, which presumably accounted for the reduction in haemolymph PO levels. Thus, there is growing evidence that this potential trade-off may occur across several insect taxa.

Selection also revealed life-history trade-offs that were not apparent when examining genetic correlations between traits (Cotter *et al.* 2004b). Melanism occurs in this species in response to population density and is associated with parasite resistance (Wilson et al. 2001), its facultative expression suggesting that it is costly. In this study, we have shown that rather than melanism itself, it is investment in PO and haemolymph protein that is costly, resulting in a slower growth rate and later emergence. There is evidence from this species that larvae in the high-density, gregarious phenotype forage on different host plants to those exhibiting the solitarious phenotype (Simmonds & Blaney 1986). It is possible, therefore, that by preferentially feeding on protein-rich plants, the high-density larvae may be able to

ameliorate these costs (Lee *et al.* 2006). Future studies examining the role of diet in the modulation of the costs of the immune response would be informative.

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Figure legends

Fig. 1 – The correlation between the quantitative spectrometer reading for larvae from each colour category and the qualitative categorical colour score. Inset: Typical larvae from each of the seven colour categories to which larvae were assigned. XD – extra dark, D – dark, DI – dark intermediate, I – intermediate, PI – pale intermediate, P – pale, XP – extra pale.

Fig. 2 – The response to selection on cuticular colour over 16 generations

a) The change in the mean colour score of the selected lines over 16 generations of selection. The selected lines were colour scored each generation. The unselected controls were scored at generations 0, 7 and 12. b) The response to selection (R) is plotted against the cumulative selection differential (S). The slope of the regression line (R/S) is equal to the realised heritability for the trait (Falconer & Mackay 1996). **1D**: y = 61.3 +0.14x, **2D**: y = 59.2 + 0.22x, **1P**: y = 66.2 - 0.21x, **2P**: y = 67.6 - 0.20x

Fig. 3 – The effect of selection on haemolymph PO activity

The predicted mean values from the REML model for each of the four selected lines, without larval weight included as a covariate: a) larval weight; b) haemolymph protein levels; c) haemolymph PO activity; and d) lysozyme. *** $P < 0.001$, * $P < 0.05$, ns $P > 0.05$

Fig. 4 – Life history traits in the selected lines

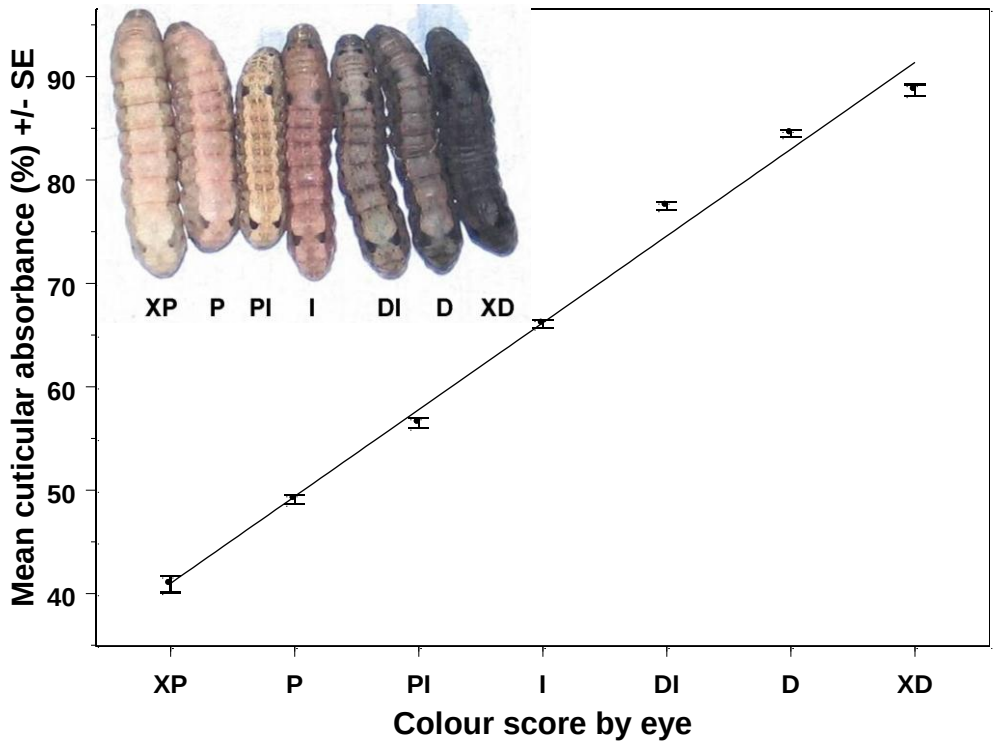
The predicted mean values from the REML model for each of the four selected lines: a) time to pupation in days; b) pupal weight in mg; c) growth rate in mg per day; and d) time to emergence in days. Comparisons in each case were made within each replicate. *** $P < 0.001$, ns $P > 0.05$

Fig. 5 – The correlation between cuticular melanisation and immune function traits.

a) The correlation between haemolymph PO levels and cuticular melanisation both within (solid lines) and across lines (dotted line) and b) the correlation between lysozyme activity and cuticular melanisation both within (solid lines) and across lines (dotted line).

1 **Figures**

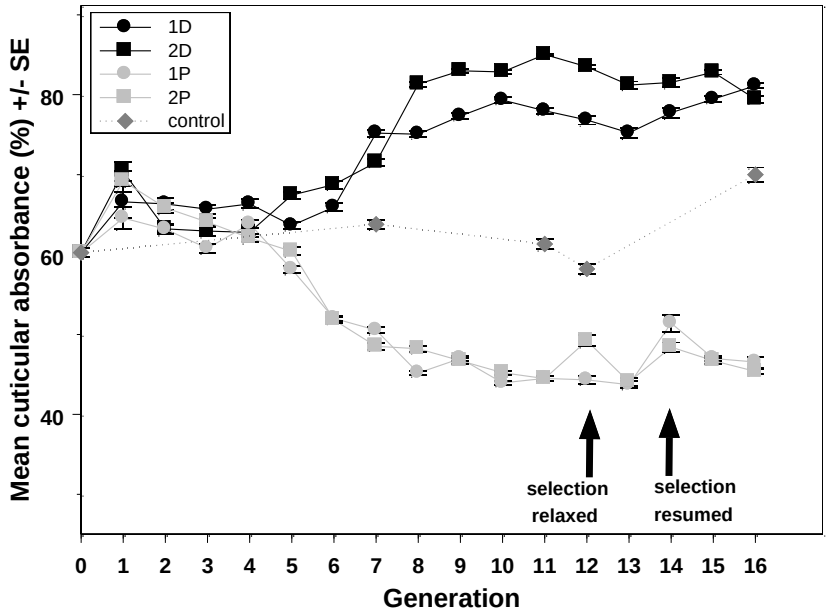
2 **Figure 1**



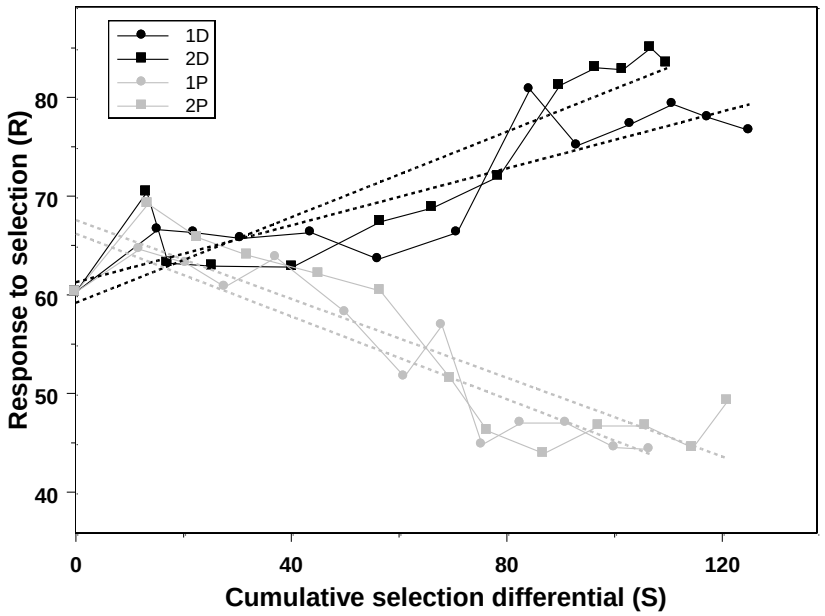
3

1 **Figure 2**

a)

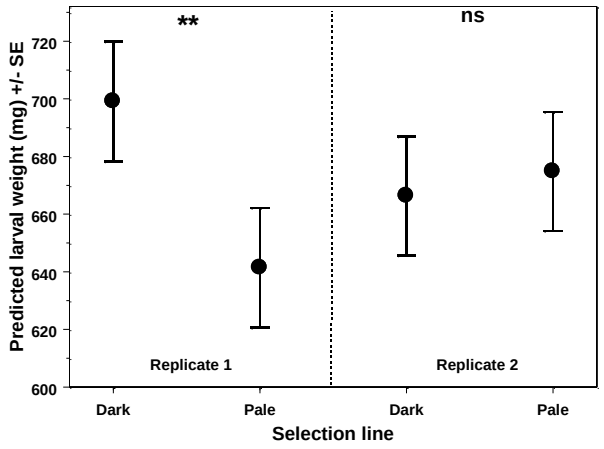

1D
2D
1P
2P
control
Mean cuticular absorbance (%) +/- SE
80
60
40
selection relaxed
selection resumed
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Generation

b)


1D
2D
1P
2P
Response to selection (R)
80
70
60
50
40
0
40
80
120
Cumulative selection differential (S)

1 **Figure 3**

a)



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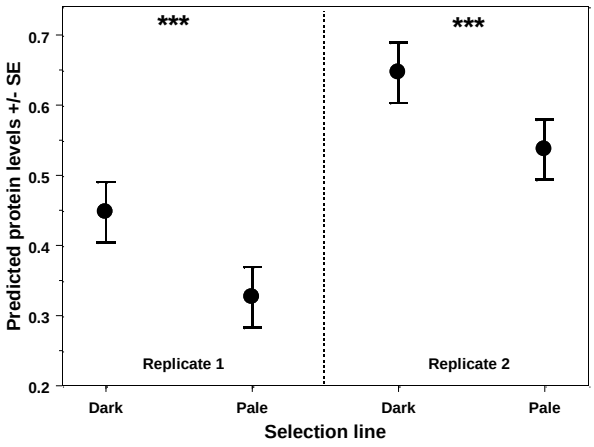

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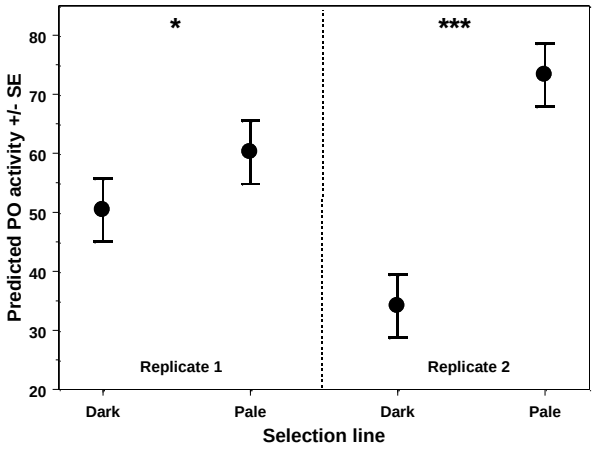
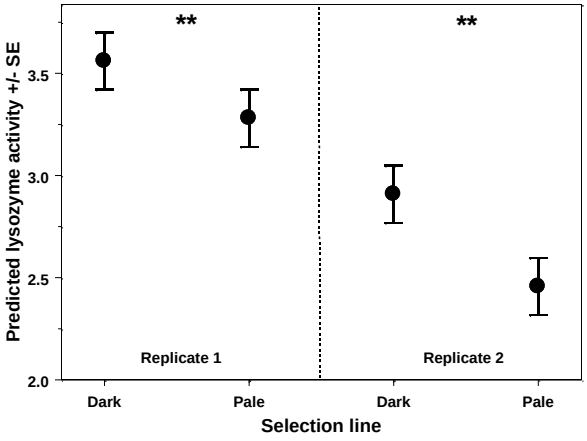


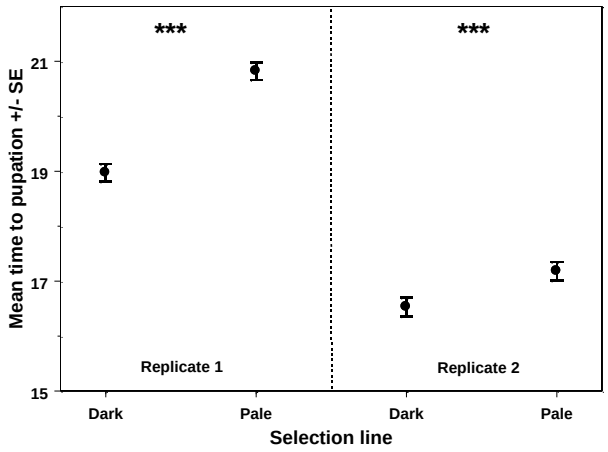
d)



2

Figure 4

a)



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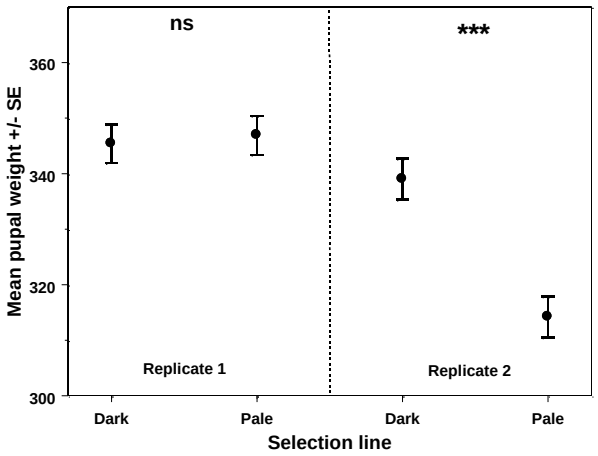

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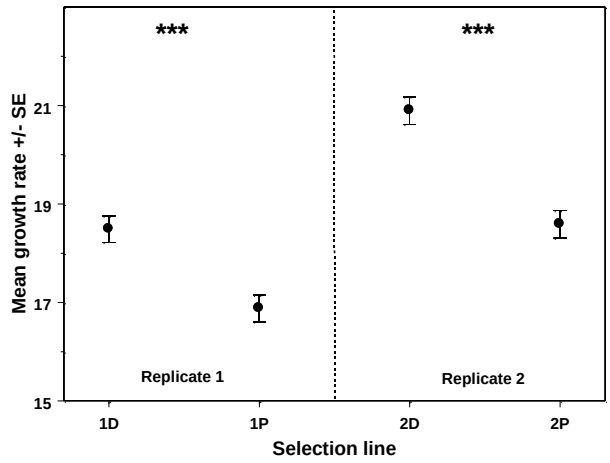
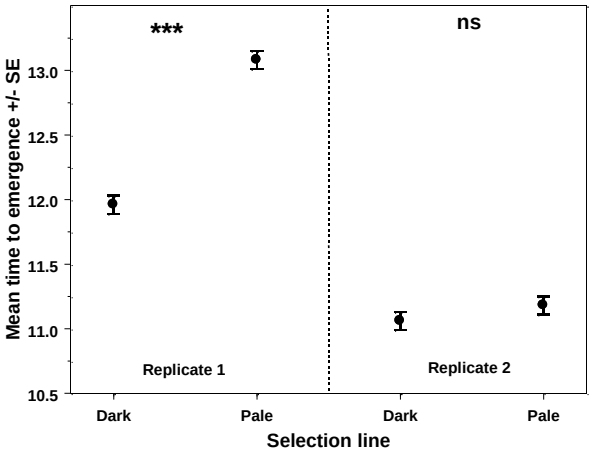
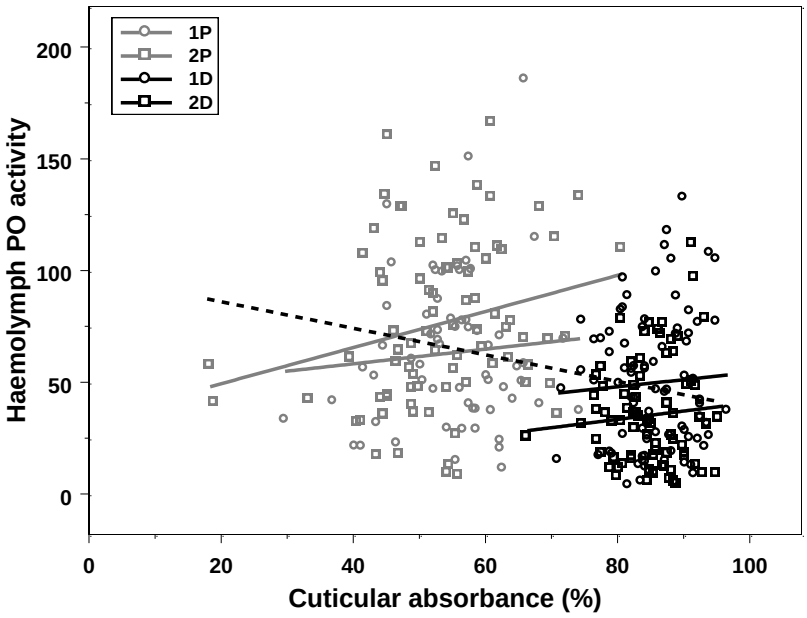


d)

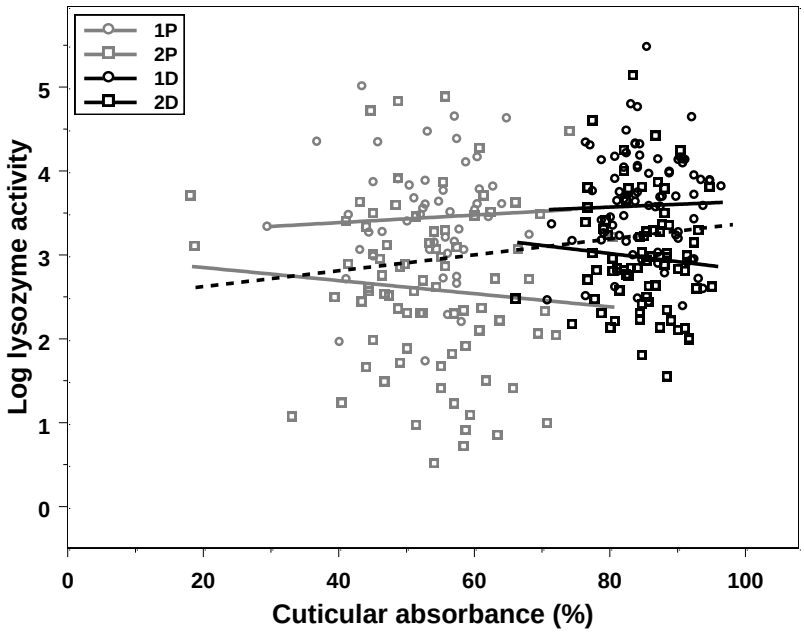


1 **Figure 5**

a)



b)



2