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Shell colour polymorphism, injuries and immune defense in three helicid snail species, *Cepaea hortensis*, *Theba pisana* and *Cornu aspersum maximum*

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ABSTRACT

Shell colour polymorphism is a widespread feature of various land snail species. In our study we aimed at elucidating the question whether there is a correlation between shell colouration and immune defense in three land snail species by comparing phenoloxidase (PO) activity levels of different morphs after immunostimulation via Zymosan A-injection. Since phenoloxidase is involved both in immune defense as well as in melanin production, the PO activity level is particularly interesting when trying to resolve this question. Even though Zymosan A failed to induce PO activity rendering a comparison of inducible PO activity impossible, an interesting difference between pale and dark morphs of all tested species could be observed: dark snails were less affected by hemolymph withdrawal and were able to maintain or regenerate a significantly higher PO activity level after hemolymph withdrawal than pale snails. Possible implications of this observation are discussed.

1. Introduction

Shell colour polymorphism is a phenomenon which can be found in several land snail species [2,19,20,37], including *Cepaea hortensis* (O.F. MÜLLER, 1774), *Theba pisana* (O.F. MÜLLER, 1774) and *Cornu aspersum* (O.F. MÜLLER, 1774). Quite a number of studies attribute this polymorphism to predation or climatic effects [23,33,32,36,37], whereas climatic effects are often explained by a higher warming capacity in darker morphs furthering the paler morphs in sun-exposed, warmer habitats [31,38]. However, recent work [52] has shown that the assumption of a higher warming capacity in darker snail shells should be regarded with caution, and alternative possibly selecting factors and correlations have been proposed, among which are humidity and the higher occurrence of parasites under humid conditions in northern and/or sheltered habitats. And in fact, it was demonstrated that wetter conditions can further parasite stress on molluscs [45]. Interestingly, a correlation between shell colouration and parasitic load was observed in several snail species with the darker morphs being less parasitized than paler morphs [13,14]. Taken together, this raises the questions whether there is a difference in pathogen resistance between dark and pale snail morphs, and which mechanism would be underlying such a correlation between shell colouration and immune defense.

A correlation between colouration and phenoloxidase (PO)-mediated immunity has already been demonstrated in insects, whereas stronger melanisation and darker cuticle colour are linked to higher immunity [4,9,22]. In fact, it is known that melanism and immunity parameters are both based on the melanin-producing pathway, the so-called PO-cascade [51,58]. This cascade can be activated via β-1,3-glucans, peptidoglycans and lipopolysaccharides, which are derived from fungi or bacteria [58]. Such β-1,3-glucans can be found, for example, in Zymosan A, a yeast cell wall preparation that is commonly used for artificial PO activity stimulation in invertebrates [59], and which was also chosen for immunostimulation in this study.

To our knowledge, nothing is known about mechanisms underlying possible links between shell colouration and immunocompetence in molluscs even though hints to such links were found some decades ago [13,14]. However, melanin has been shown to be a pigment which is also responsible for colouration of snail shells [18], and PO is an important parameter in immune defense against microbial and parasitic pathogens in molluscs [1,6], also playing an important role in wound healing [49] and sclerotization of molluscan shells [47,61]. Therefore it is imaginable that correlations between shell pigmentation and immune defense and between shell pigmentation and wound healing/sclerotization processes exist in molluscs as well.

In this study, we have focused on investigating correlations between shell colouration and constitutive as well as inducible PO activity in different shell colour morphs of *Cepaea hortensis*, *Theba pisana*

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and *Cornu aspersum maximum*, applying Zymosan A as an immunostimulant.

2. Material and methods

2.1. Test organisms, sampling and maintenance

Tests were conducted with three different land snail species, *Cepaea hortensis* (O.F. Müller, 1774), *Theba pisana* (O.F. Müller, 1774) and *Cornu aspersum maximum* (cultivated variety of *Cornu aspersum*, O.F. Müller, 1774; nomenclature according to Falkner et al. [27]). *Cornu aspersum maxium* was obtained from a local snail farm (Schneckengarten Munderkingen, Munderkingen, Germany) and acclimatised to laboratory conditions (20 °C, 60–90% humidity) for at least two weeks before the experiments were started. *Cepaea hortensis* was sampled from a private garden in Tübingen-Lustnau, Germany, where no pesticides are applied, and *Theba pisana* was collected near Les Paluds de Noves (Dept. Bouches du Rhône) in Southern France. Before testing, *C. hortensis* and *T. pisana* were acclimatised to laboratory conditions (18 °C for *C. hortensis*, 22 °C for *T. pisana*, 50–90% humidity) for at least two weeks. All snails were fed a diet of organic carrots/cucumbers/zucchini/oats *ad libitum* once a week and organic baby food (*Hipp Bio-Milchbrei*, Hipp GmbH & Co. Vertrieb KG, Pfaffenhofen, Germany), prepared according to package instructions, *ad libitum* twice a week. Clean cuttlebone was provided *ad libitum* at all times. Animals were kept in ventilated plastic terraria (30 × 19.5 × 20.5 cm^3) containing a moistened 2 cm layer of *JBL Terra Basis ground covering for terraria* (JBL GmbH & Co. KG, Neuhofen, Germany). Terraria were re-moistened with tap water every other day and cleaned on a weekly basis.

2.2. Experimental set-up, general

To avoid possible bias through naturally existing nematode infections, 10% of the sampled snails were tested by peptic digestion of pieces of the headfoot as described in Cabaret [15]. In all cases, no nematodes could be detected.

In all tests the snails were individually exposed to the respective (mentioned below for each specific test) exposure or control conditions in plastic boxes (9 × 6 × 9 cm^3, with perforated transparent lids, in case of *C. hortensis* and *T. pisana*) or plastic terraria (16.5 × 16.5 × 19 cm^3) with lid and wall perforations, in case of C. *aspersum maximum*) lined with a moistened 2 cm layer of *JBL Terra Basis ground covering for terraria* (JBL GmbH & Co. KG, Neuhofen, Germany). The species-specific laboratory maintenance temperatures as mentioned above were sustained throughout the respective tests. Hemolymph collection was conducted according to Renwrantz et al. [50], with the following modifications: hemolymph (HL) was withdrawn from each animal from the hemocoel of the upper to middle subepithelial region of the headfoot at a quantity of 20 μL using sterile syringes (1 mL) and 0.40 × 20 mM gauge sterile hypodermic needles. In *C. aspersum maximum*, pre-drilling of a small hole into the shells was required due to the shells' hardness. Punctured veins were not glued as this resulted in strong mucus production during pre-tests.

For Zymosan A- injections the same types of syringes and needles, and the same puncture sites were used as for HL collection. The Zymosan A solution contained 5 mg Zymosan A (Sigma Aldrich Chemie GmbH, Steinheim, Germany) in 1 mL snail saline (prepared according to Chiarandini [17]), equivalent to ± 4 × 10^7 particles/mL (as in Matricon-Gondran and Letocart [44]). The Zymosan A solution was freshly prepared for each test.

Originally, we planned to re-sample Zymosan A- injected snails 6 h and 24 h after injection. The 24 h time point was chosen as it has been shown in other molluscs that PO activity can increase two-fold within 24 h after Zymosan A-injection [1]. The 6 h time point was chosen in order to test for a possibly earlier PO activity induction.

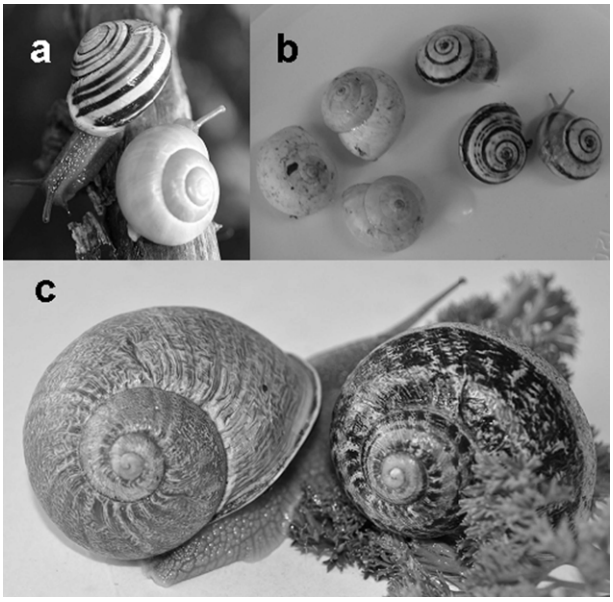

Fig. 1. Different morphs of test snails; pale and dark morph of *C. hortensis* (a), pale and dark morphs of *T. pisana* (b), and pale and dark morph of *C. aspersum maximum* (c).

However, in *C. hortensis* and *T. pisana*, amendments to this schedule were necessary as described below.

Experimental set-up for C. hortensis

Snails were divided in two different morph groups: yellow (later referred to as 'pale' (p)) and strongly-banded with five brown bands on yellow base colour (later referred to as 'dark' (d)) (Fig. 1a). Of each morph group, 14 animals were sampled for HL collection at the beginning of the experiment (0 h, base level). Then 100 μL of the Zymosan A- solution were injected into each snail. After 24 h, snails were sampled for HL collection again (24 h Zymosan A exposure). In contrast to our later experiments with *C. aspersum maximum*, we avoided sampling hemolymph at 6 h of test time, as this proved to be too stressful for *C. hortensis* in pre-tests, probably due to the short recovery time between 0 h and 6 h, and the relatively small size of *C. hortensis*.

2.3. Experimental set-up for T. pisana

Snails were divided in two different morph groups: pale white (referred to as 'pale' (p) in the following) and darkly- banded with distinct, dark- brown bands (referred to as 'dark' (d) in the following) (Fig. 1b). Tests were conducted in two runs for each morph: (1) 10 animals were sampled for HL collection at 0 h (base level 1). After 24 h, these animals were resampled for a further HL collection (24 h HL withdrawal). (2) Ten animals were sampled for HL collection at 0 h (base level 2). These animals were also injected an 100 μL aliquot of the Zymosan A solution each after the 0 h-HL collection. After 24 h, these snails were resampled for HL collection (HL withdrawal + 24 h Zymosan A exposure). Another 10 snails were injected 100 μL of Zymosan A solution each at 0 h without prior HL collection. After 24 h, they were sampled for HL collection (24 h Zymosan A exposure). As in *C. hortensis*, we omitted a 6 h hemolymph-sampling due to the short time span between 0 h and 6 h, and the small size of the snails.

2.4. Experimental set-up for C. aspersum maximum

Snails were divided in two different morph groups: pale brownish/yellowish without bands (referred to as 'pale' (p) in the following) and dark brown with bands (referred to as 'dark' (d) in the following) (Fig.

1c). Tests with this species were also conducted in two runs for each morph: (1) 15 snails were sampled for HL collection at 0 h, 6 h and 24 h (base level 1, 6 h HL withdrawal and 24 h HL withdrawal, the latter as corresponding controls to the respective Zymosan A exposure times). Another 15 animals were sampled for HL collection at 0 h (base level 2), these animals were also injected 100 μL of the Zymosan A solution each at 0 h. They were resampled at 6 h and 24 h (HL withdrawal + 6 h and 24 h Zymosan A exposure). (2) Fifteen animals were sampled for HL collection at 0 h (base level 3). Another 15 animals were sampled for HL collection at 6 h (6 h control), these animals were resampled at 24 h (24 h control). A further 15 snails were injected an 100 μL aliquot of the Zymosan A solution each at 0 h without prior HL collection. These animals were sampled for HL collection at 6 h (6 h Zymosan A exposure) and 24 h (24h Zymosan A exposure).

2.5. Phenoloxidase (PO) assay

The phenoloxidase assay was conducted with hemolymph (HL) samples. This restriction to HL samples was chosen as we aimed at depicting the immune-defense-related function of PO as a part of the humoral immune response of molluscs (as described in Gliński and Jarosz [28]), avoiding possible bias through, for example, reproduction-related functions of the enzyme in other tissues [7,40]. Furthermore, the analysis of PO in HL samples has already been successfully performed for a number of invertebrate species, including molluscs [10,39,46,53,54,56], and PO analysis in other tissue samples is rather regarded as an alternative in case HL collection is not feasible [43]. The assay procedure was adapted from Seppälä and Jokela [54] with slight modifications, and care was taken to prevent unwanted unfolding of hemocyanin [26,35]. In short, 20 μL of hemolymph (HL) were mixed with 200 μL of phosphate buffered saline (PBS, pH 7.4; Sigma Aldrich Chemie GmbH, Steinheim, Germany) and immediately shock-frozen in liquid nitrogen. The resulting samples were stored at −80 °C until further processing. After thawing, 40 μL sample aliquots were placed in 96-well microtiter plate wells which contained 140 μL of cold aqua bidest. and 20 μL of PBS each. Each sample was measured in triplicates. Additionally, four controls (sample aliquots replaced by aqua bidest.) per plate were set up. Then, 20 μL of cold L-dopa (Sigma Aldrich Chemie GmbH, Steinheim, Germany) solution (4 mg/mL aqua bidest.) were added to each well and plates were immediately measured photometrically at 490 nm in a microplate reader (ELx800, Bio-Tek Instruments, INC., Vermont, USA) resulting in 0 h values. The plates were then covered and incubated at 30 °C in a thermocabinet (ST 2 A60, STL-Neckarwestheim, Neckarwestheim, Germany) for a species-specific time (30 min for *C. hortensis*, 6 h for *T. pisana* and 3.5 h for *C. aspersum maximum*, determined in pre-tests, data not shown) to ensure linearity of the absorbance increase allowing most accurate measurments. After incubation, the plates were re-measured photometrically at 490 nm resulting in incubation time values. PO activity was then calculated according to the following equation:

$$\text{PO activity} = \text{incubation time values} - \text{0h values} - \text{mean absorbance change in controls},$$

and expressed in milliunits.

2.6. Statistical analysis

The obtained data were statistically analysed implanting JMP® 9.0 (SAS Institute Inc., Cary, USA). Data were tested for normality using the Shapiro–Wilks-test, and when the following normal distribution was analysed via Tukey–Kramer-HSD for significant differences. Not normally distributed data were analysed for significant differences using the non-parametric Wilcoxon *U*- test and in case of multiple comparisons, a Bonferroni correction was applied. Levels of significance were set to $0.01 < p \leq 0.05$: *; $0.001 < p \leq 0.01$: **; $p \leq 0.001$: *** for normally distributed data and not normally distributed data used in single comparisons. For not normally distributed data analysed in multiple comparisons, the levels of significance were calculated implanting a Bonferroni correction; they are shown in the respective figure legends.

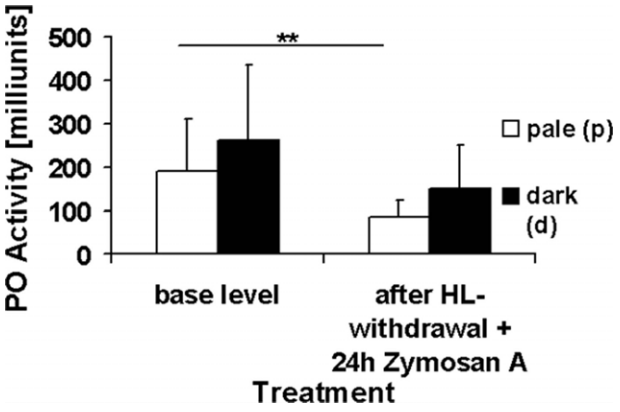

Fig. 2. Phenoloxidase (PO) activity levels in different morphs of *C. hortensis*; base levels and levels after hemolymph (HL) withdrawal and 24 h Zymosan A-exposure (mean + sd; $n = 10$; $0.001 < p \leq 0.01$: **).

3. Results

3.1. Phenoloxidase (PO) activity in C. hortensis

The base levels of the different morphs were not significantly different from each other, even though the level of dark morphs tended to be higher (Fig. 2). Also, the PO activity levels of the different morphs after HL withdrawal and Zymosan A exposure for 24 h did not differ significantly (Fig. 2). However, when comparing the results within the morph groups it became evident that the PO activity level in pale morphs decreased significantly after HL withdrawal and Zymosan A exposure for 24 h (Fig. 2). In the dark morphs no significant difference could be detected between base level and results after HL withdrawal and Zymosan A exposure for 24 h (Fig. 2).

3.2. Phenoloxidase activity in T. pisana

Run 1: No significant differences between the base levels (base level 1) were found between the two morphs, yet there was a tendency for a higher level in dark morphs (Fig. 3). Twenty four hour after HL withdrawal no significant differences between the two morphs could be detected as well (Fig. 3). However, the 24 h HL withdrawal results for the pale morphs had significantly decreased compared to the respective base level, whereas no such decrease could be found in the dark morphs (Fig. 3).

Run 2: When comparing the two morphs, no significant differences were found concerning the base levels (base level 2) even though, by trend, the level in dark morphs appeared higher, also there were no significant differences detectable between the base levels of run 1 and run 2 (Fig. 3). The HL withdrawal combined with a 24h Zymosan A exposure resulted in a significant decrease of the PO activity level compared to the base level in both morphs (Fig. 3). A 24 h Zymosan A exposure without prior HL withdrawal did not result in significant differences compared to the base levels in both morphs (Fig. 3). However, in both morphs the 24 h Zymosan A exposure results were significantly higher compared to the respective HL withdrawal + 24 h Zymosan A exposure results (Fig. 3). Furthermore, the 24 h Zymosan A exposure data recorded for the two morphs differed significantly from each other with the dark morphs showing a higher PO activity level.

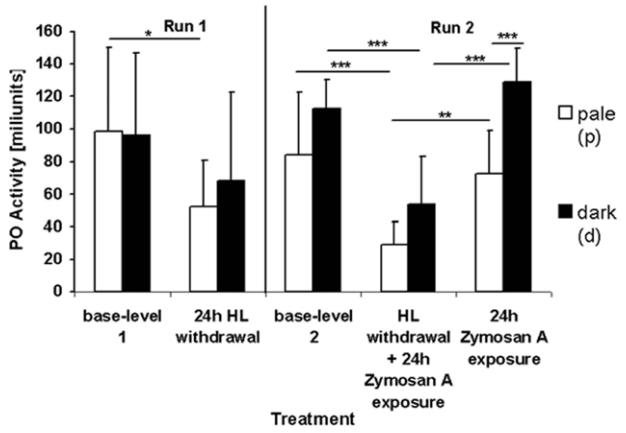

Fig. 3. Phenoloxidase (PO) activity levels in different morphs of *T. pisana*; test run 1 with base levels and levels 24 h after hemolymph (HL) withdrawal, and test run 2 with base levels, levels after hemolymph withdrawal and 24 h Zymosan A-exposure and levels after 24 h Zymosan A-exposure only (mean + sd; $n = 10$; $0.01 < p \leq 0.05$: *; $0.001 < p \leq 0.01$: **; $p \leq 0.001$: ***).

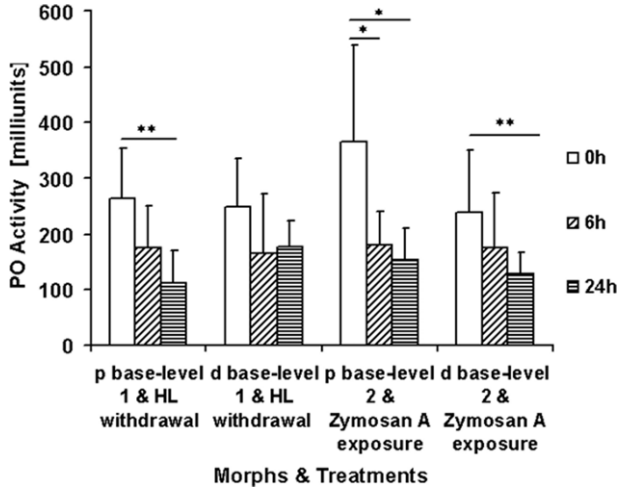

Fig. 4. Phenoloxidase (PO) activity levels in different morphs of *C. aspersum maximum*; test run 1 with base-levels and levels 6 h or 24 h after hemolymph (HL) withdrawal considering the same individuals as for the base levels, and base levels and levels after hemolymph (HL) withdrawal plus 24 h Zymosan A-exposure considering the same individuals as for the base levels (mean + sd; $n = 15$; $0.000435 < p \leq 0.0022$: *; $p \leq 0.000435$: ** after Bonferroni-corrections for 23 comparisons).

3.3. Phenoloxidase activity in C. aspersum maximum

Run 1: The base levels (base level 1) of the two morphs did not differ significantly from each other (Fig. 4). Also, the 6 h HL withdrawal results were not significantly different between the two morphs, and did not differ from their respective base levels as well (Fig. 4). However, the 24 h HL withdrawal results in the pale morphs showed a significant decrease compared to the respective base level, whereas no such difference was found in the dark snails (Fig. 4). The base levels 2 of the different morphs did not differ from each other as well. Also, there were no significant differences between base levels 1 and 2 (Fig. 4). HL withdrawal combined with a 6 h Zymosan A exposure resulted in a significantly decreased PO activity level for the pale morphs but not for the dark morphs (Fig. 4). After HL withdrawal + 24 h Zymosan A exposure the PO activity levels were significantly decreased in both morphs (Fig. 4).

Run 2: In run 2, no significantly different results could be detected, only a trend for slightly higher levels in base level and control snails was observable for the dark morphs (Fig. 5).

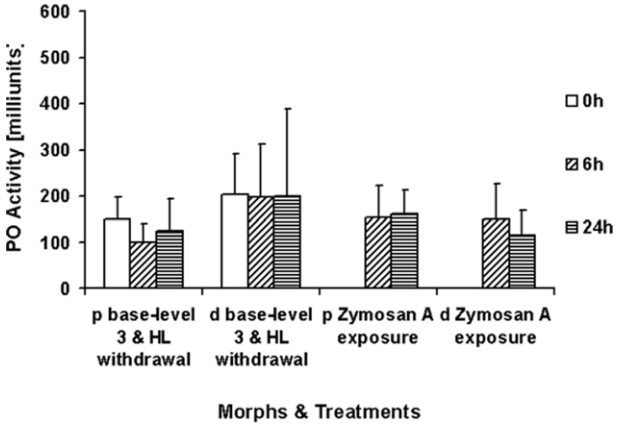

Fig. 5. Phenoloxidase (PO) activity levels in different morphs of *C. aspersum maximum*; test run 2 with base levels and levels 6 h or 24 h after hemolymph (HL) withdrawal considering other individuals as for the base levels, and levels after 6 h or 24 h Zymosan A-exposure (mean + sd; $n = 15$; $p \leq 0.0024$:* after Bonferroni-corrections for 21 comparisons).

4. Discussion

Concerning the constitutive levels (base levels) of PO activity, it is remarkable that we did not find significant differences between different morphs in each of the species tested. This is contrary to what has been found in other invertebrate species, e.g. *Tenebrio* beetles [4], or other insects [62]. These studies revealed a positive relation between either stronger melanisation and higher constitutive PO activity levels leading to increased pathogen resistance in darker animals [4], or between melanism and disease resistance involving phenoloxidase [62]. However, concerning the three snail species tested in our study, we may exclude a higher pathogen resistance of darker snails based on differences in constitutive PO activity levels. A possible explanation for the lack of differences in the constitutive PO activity levels of different morphs is the differential structure of hemocyanins, which also exhibit PO activity and have immunological functions [25], in different molluscs as it was demonstrated by De Smet et al. [24] and Velkova et al. [60]. The stability of snails' hemocyanin due to possession of three structural subunits compared to two subunits only in other molluscs [24,60] can account for the absence of detectable differences in constitutive levels in snails. Another explanation comes from the fact that maintaining relatively high constitutive (or prophylactic) levels of phenoloxidase can provide animals with the benefit of higher resistance to pathogens, yet this may also be costly for the respective organisms [55]. It is also known that phenoloxidase activity provides cytotoxic properties, this has, for example, been observed in ascidians [8] and is based on the generation of reactive oxygen metabolites during the PO-mediated conversion of phenols to *o*-quinones and then melanin. Therefore it is likely that maintaining a high constitutive PO activity level poses a considerable oxidative stress on the respective organism, in our case snails. A solution to the 'dilemma' of oxidative stress vs. pathogen resistance might come from relying on induced PO activity as it has been proposed for lighter-coloured beetles by Armitage and Siva-Jothy [4], this restricts oxidative stress deriving from PO activity to periods of actual demand for immune response. However, in our experiments with *C. hortensis*, *T. pisana* and *C. aspersum maximum*, we did not observe any upregulation of PO activity following injection of Zymosan A in any of these three species. Consequently, no differences in inducible PO activity could be observed in different morphs. This might lead to the exclusion of a higher pathogen resistance based on different PO activity in darker morphs, rejecting our hypothesis. Yet, it has to be taken into account that the fact that the Zymosan A-injection failed

to induce higher PO activity levels in all three species should be considered a rather unusual phenomenon. Immunostimulation via β-1,3 glucans, being a major component of yeast cell walls (Zymosan A), has been observed in a variety of invertebrates [16,59,48], including molluscs [1,21,34,41]. However, there is also work showing that Zymosan A can appear ineffective in causing elevated PO activity levels [3,11], yet studies on this phenomenon are relatively scarce and no concluding explanation for this has been offered so far. Yet, an observation of Brivio et al. [11] was that increasing Ca^{2+} concentration led to decreasing PO activity in the hemolymph of their test organisms (*Allogamus auricollis*), and they suggest that high calcium levels may protect against unwanted proPO activation in insects as this is also known for other arthropods [5,57]. As calcium ion levels in snail hemolymphs are relatively high [29,30], it is possible that a high calcium content might also form a barrier against undesired PO activity in our test snails. Again, although being a plausible explanation for the absence of Zymosan A-related PO activity increase in our snails, we cannot draw a final conclusion from this ruling out other, probably unknown factors that might have contributed to or even exclusively led to the observed phenomenon of PO activity decline. As a consequence, we find it difficult to conclude whether darker morphs of the three test species possess a stronger immunocompetence based on PO activity than paler conspecifics or not.

However, we did observe a remarkable difference between dark and pale morphs in all three tested snail species: 24 h after hemolymph-withdrawal the PO activity level was significantly decreased in the hemolymph samples of pale snails but not in the samples of dark animals. As this probably affected a possible PO activity induction due to Zymosan A, we repeated the experiments with *T. pisana* and *C. aspersum maximum* without prior hemolymph-withdrawal (run 2) to exclude bias through this, yet no Zymosan A-related PO activity increase was observable as discussed above. A plausible explanation for the significant decrease of PO activity following hemolymph withdrawal solely in pale snails could be that pale snails possibly cannot compensate for hemolymph and/or hemocyte loss as fast or as effectively as dark snails. As PO is produced by hemocytes [12], and positive correlations between hemocyte density and PO activity levels have been found in insects as well as molluscs [22,54], it is possible that hemolymph-withdrawal causing a reduction of hemocyte numbers also results in reduced PO activity. Taking this into account, our results indicate that dark snails can either regenerate hemocyte numbers or PO content in the hemolymph within 24 h to a better extent than pale snails. One might also presume that hemocyte numbers were generally higher in dark snails, however, the absence of significantly different constitutive levels in different morphs contradicts this. As hematopoiesis in gastropods is generally only poorly understood [42], we find it difficult to speculate on possible mechanisms underlying such a regeneration of hemocyte numbers/hemolymph PO content and its plausible links to colour polymorphism in land snails, and recommend further investigations on this topic implanting corresponding hemocyte counts. Nevertheless, considering our results it is plausible that dark snails benefit from being obviously less affected by hemolymph withdrawal concerning PO activity levels than pale snails. Such a benefit can, for example, occur following injuries involving shell and/or tissue impairment leading to hemolymph loss and requiring wound healing processes. This consideration gains in importance when taking into account that phenoloxidase plays an important role in shell sclerotization of molluscs [47,61]. As phenoloxidase is an important parameter in pathogen resistance in molluscs [12,34], and as tissue and/or shell impairment can facilitate pathogen infections since this affects the main physical barriers of molluscs [28], it becomes even more plausible that darker snail morphs can be at an advantage compared to paler morphs when shell and body wall injuries occur.

Even though, due to lack of induction via Zymosan A, no differences in PO activity levels in different morphs could be observed in this experiment, another difference between pale and dark snail morphs concerning their immune response to hemolymph withdrawal stress was detected in our study. This is interesting and implies that the dark morphs benefit under certain circumstances from being able to regenerate or preserve hemolymph PO content to a better extent than pale morphs.

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