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Shell colouration and parasite tolerance in two helicoid snail species

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ABSTRACT

The polymorphism of shell colouration in helicoid snails is a well-known phenomenon attributed to different factors such as predation and climatic effects. Another aspect contributing to this polymorphism could be the interplay of melanin production and phenoloxidase-related immunity. Therefore, in this study we aimed at answering the questions whether there is a differential sensitivity of different snail shell colour morphs to nematode infection, and whether this can be related to differences in phenoloxidase (PO) activity levels using the two helicoid, polymorphic snail species *Cepaea hortensis* and *Cernuella virgata*. Snails of both species were artificially infected with the parasitic nematode *Phasmarhabditis hermaphrodita*, and analysed for mortality and PO activity levels. We found *C. virgata* to be more severely affected by *P. hermaphrodita* infection than *C. hortensis*, and the dark *C. virgata* morphs to be more resistant to lethal effects of this infection than pale morphs. However, these differences in sensitivity to the parasite could not clearly be related to different PO activity levels.

1. Introduction

Shell colour polymorphism is a phenomenon widely observed among several land snail species (Cook, 1986; Goodfriend, 1986), including helicoid snails such as *Cepaea hortensis* (O.F. Müller, 1774) and *Cernuella virgata* (Da Costa, 1778) (Jones et al., 1977; Gittenberger, 1993). This phenomenon has been related to a number of different factors, namely predation and climatic effects (Cowie, 1990; Heller, 1981; Heller and Gadot, 1984; Jones et al., 1977; Johnson, 2011, 1981). However, recent work has shown that the idea of a different warming capacity in differently coloured morphs, which has been used to explain climatic effects, should be regarded with caution and probably does not apply to snail species with a constant primary shell colour and just different banding patterns (Scheil et al., 2012a). Furthermore, it has been observed that under wet climatic conditions parasite stress is higher on snails than in dry climate as both parasite and snail development will benefit from wet ground conditions, and snail activity increases under wet conditions furthering parasite-snail encounters (Morley and Lewis, 2008). Additionally, it was found that in some helicid snails pale, unbanded morphs are more severely parasitized by nematodes than banded morphs (Cabaret, 1988, 1983; Lahmar et al., 1990), thus leading to the hypothesis that wet climate might favour dark morphs. A higher pathogen resistance of darker morphs has also been observed in other invertebrates previously, e.g. in insects (Armitage and Siva-Jothy, 2005; Barnes and Siva-Jothy, 2000; Cotter et al., 2004). It has been shown as well that this kind of resistance in insects is based on higher phenoloxidase (PO) levels in the hemolymph of dark morphs (Armitage and Siva-Jothy, 2005; Wilson et al., 2001), and that in invertebrates both melanism and immunity share the melanin-producing pathway, the so-called PO-cascade (Rolff and Siva-Jothy, 2003; Söderhäll and Cerenius, 1998). A stimulation of PO activity following injection of non-self molecules has been demonstrated in bivalves (Hellio et al., 2007), and PO activity has already been used as an immune parameter in a variety of different molluscs (Bahgat et al., 2002; Barracco et al., 1999; Jordan and Deaton, 2005; Munoz et al., 2006; Seppälä et al., 2011; Seppälä and Jokela, 2010; Smith and Söderhäll, 1991). However, in snails, to our knowledge, no studies on a possible correlation between colour polymorphism and parasite tolerance including analysis of the supposably underlying parameter PO activity have been conducted so far, even though it is known that melanin is a pigment accountable for molluscan shell colouration (Comfort, 1951). Hence, in this study, we chose to investigate the effects of the parasitic nematode *Phasmarhabditis hermaphrodita* (A. Schneider, 1859), a nematode which is considered widespread in Europe (Rae et al., 2007) and also commercially available for slug and snail control (Nemaslug®, *Becker Underwood, Littlehampton, UK*), on mortality and PO activity levels in the two helicoid snail species *C. hortensis* and *C. virgata*. We aimed at shedding some light on the following questions: Is there a differential tolerance of

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different morphs of these species to *P. hermaphrodita* and, if so, can this be explained by differences in PO activity?

2. Materials and methods

2.1. Test Animals and laboratory maintenance

Adult *C. hortensis* were collected from meadows and shrubs near the rail tracks of Tübingen–Derendingen, Baden–Württemberg, Germany, after nightly rainfalls in June, 2011. Two morphs, a yellow unbanded morph (later referred to as 'pale') and a yellow banded morph with five distinct brown bands (later referred to as 'dark') were sampled. *C. virgata* (adult individuals) were sampled 5 km south of Volterra, Tuscany, Italy, on shrubs, in August 2011. Again, two morphs were sampled, a pale white morph without banding (later referred to as 'pale'), and a morph with distinct brown banding (later referred to as 'dark'). For both species, only individuals clearly belonging to one morph group (no intermediates) were employed.

Both species were kept in ventilated plastic terraria (30 cm × 18 cm × 19 cm; Exoterra medium, Hagen Deutschland GmbH & Co. KG, Holm, Germany) laid-out with a 2 cm cover of moistened JBL Terra Basis ground covering for terraria (JBL GmbH & Co. KG, Neuhofen, Germany). The ground covering was re-moistened with tap water every other day and terraria were thoroughly cleaned at weekly intervals. The snails were fed an ad libitum diet of organic carrots, zucchini, oats and cucumbers once a week, and organic baby food (Hipp Bio-Milchbrei, Hipp GmbH & Co. Vertrieb KG, Pfaffenhofen, Germany) prepared according to package instructions twice a week. Clean cuttlebone was provided at all times. The snail terraria were installed in climate chambers and snails acclimatized to laboratory conditions (18 °C for *C. hortensis*/22 °C for C. virgata; 50–90% humidity; 12 h:12 h light–dark cycle) for two weeks at least before starting experiments.

2.2. Experimental design and sampling

For experimental infection of snails, *P. hermaphrodita* (Rhabditida, Nematoda) were obtained commercially as Nemaslug® (*Becker Underwood, Littlehampton, UK*) from a local distributor (*Sautter & Stepper, Ammerbuch, Germany*). For the PO assay experiment, the snails were individually infected using the following method: Nemaslug® was diluted in temperature-equilibrated (18 °C) tap water to the respective nominal nematode concentrations (Table 1). Since individual infections were shown to be more successful than mass-infections in other snails (Sauerländer, 1979) we applied the Nemaslug® solution via the shell opening to each snail allotted for infection. During this process snails were individually fixed to the bottoms of the test boxes (9 × 6 × 9 cm) for 15 min using adhesive gum (*Reusable Adhesive Gum, Lyreco, Barsinghausen, Germany*). Subsequently, snails were freed from fixation and the plastic boxes filled with a 2 cm layer of moistened ground covering. Additionally, the ground covering was infused with Nemaslug® solution of the respective nematode concentrations in some experiments (Table 1). Control snails were left uninfected and the respective ground coverings were left uninfused. Snails were then kept as described in 2.1. At daily check-ups, snails which had attached to the lids or walls of the test boxes were placed back onto the ground coverings.

Hemolymph (HL) collection was conducted from the hemocoel located at the upper to middle subepithelial region of the headfoot (=anterior to middle part of the foot) of each test snail (according to the method of Renwrantz et al. (1981), with slight modifications). Hemolymph (HL) was withdrawn at a quantity of 20 µL using sterile syringes (1 mL) with 0.40 × 20 mM gauge sterile hypodermic needles. The sampled HL was 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. These HL samples were stored at −80 °C until further processing.

2.3. Phenoloxidase assays

The phenoloxidase (PO) assays were conducted using hemolymph (HL) samples. This method was chosen in favour of body tissue homogenates as we aimed at displaying the immune defense-related function of PO as part of the combined humoral and cellular (hemocyte-associated) molluscan immune defense as described in Gliński and Jarosz (1997), avoiding possible bias through reproduction-related functions of PO in other tissues (Bai et al., 1997; Kalyani et al., 1985). Additionally, the analysis of PO in HL samples has already been successfully performed in a variety of adult invertebrate species, including molluscs (Bahgat et al., 2002; Barracco et al., 1999; Hellio et al., 2007; Jordan and Deaton, 2005; Munoz et al., 2006; Seppälä et al., 2011; Seppälä and Jokela, 2010; Smith and Söderhäll, 1991), whereas the analysis of PO in tissue homogenates can be considered as an alternative for experiments on larvae or juveniles where hemolymph sampling is hardly feasible (Luna-González et al., 2003). Furthermore, considering the mode of infection and multiplication of *P. hermaphrodita* and its associated bacterium, *Moraxella osloensis*, (Glen et al., 1996, 1994; Richards et al., 2008; Tan and Grewal, 2001; Wilson et al., 1993a,b), the hemolymph of snails can be regarded as the tissue where defense reactions against the parasite are to be observed with high probability.

The PO-assays were conducted according to Seppälä and Jokela (2010) with slight modifications as in Scheil et al. (2013): After thawing on ice, 40 µL aliquots of the HL samples were placed in 96-well microtiter plate wells containing 140 µL of cold aqua bidestillata (aqua bidest., double-distilled water) and 20 µL of PBS each. Measurements were done in triplicates. Four controls (sample aliquots replaced by aqua bidest.) per plate were set up. After adding

Table 1
Exposure-scheme for PO-analysis experiments with *C. hortensis* and *C. virgata*. Base level is constitutive level at the beginning of the experiments.

Time point of HL collection	Control	Exposure
	No nematodes applied	Soil infusion approx. 3000 nematodes/box + individual application approx. 3000 nematodes/snail (≈single supplier-recommended application rate)
0 h (base level 1)	*C. hortensis* (n = 15); *C. virgata* (n = 10)	–
0 h (base level 2)	*C. hortensis* (n = 15)	–
6 h	*C. hortensis* (n = 15)	*C. hortensis* (n = 15)
24 h	*C. hortensis* (n = 15); *C. virgata* (n = 10)	*C. hortensis*(n = 15); *C. virgata* (n = 10)
3 d	*C. hortensis* (n = 15); *C. virgata* (n = 10)	*C. hortensis* (n = 15); *C. virgata* (n = 10)
5 d	*C. virgata* (n = 10)	*C. virgata* (n = 10)
7 d	*C. hortensis* (n = 15)	*C. hortensis* (n = 15)