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Autotomy reduces immune function and antioxidant defence

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Costs of autotomy, an antipredator defence, are typically explained by impaired mobility; yet physiologically mediated costs may also play a role. Given the resemblance to wounding, a decreased immune function and an associated reduction in antioxidant defence is expected after autotomy. In line with this, after lamellae autotomy, larvae of the damselfly *Lestes viridis* showed lower levels of innate immunity (i.e. phenoloxidase, PO) and antioxidant defence (superoxide dismutase, SOD). Levels of catalase (CAT) remained, however, unaffected. In line with its cytotoxicity, PO covaried positively with CAT, yet negatively with SOD. We identified a novel cost of autotomy in terms of a reduced innate immunity, which may provide an alternative explanation for the often observed costs of autotomy and which may generate indirect interactions between predators and parasites.

Keywords: antioxidant defence; antipredator defence; autotomy; damselfly larvae; ecological immunology

1. INTRODUCTION

Autotomy, the amputation of a body part, is a very widespread escape mechanism when grasped by a predator (Fleming *et al.* 2007). This antipredator defence has a clear immediate survival advantage, but also has future costs such as reduced mating success and survival, which are typically attributed to reduced locomotory abilities (Fleming *et al.* 2007). Given its resemblance to wounding, negative effects of autotomy on investment in immune function are to be expected (Plaistow *et al.* 2003). Such effects may make up an important type of previously neglected costs of autotomy. Furthermore, immune function may covary with oxidative stress due to the associated release of cytotoxic chemicals (Nappi & Christensen 2005), which may also affect fitness (Ahmad 1992).

A key component of the arthropod immune system is phenoloxidase (PO). This enzyme catalyses melanin formation, used to encapsulate pathogens, and thereby produces quinone intermediates as well as cytotoxic superoxide anions and hydroxyl radicals that participate in pathogen destruction (Nappi & Christensen 2005). As these are likely to be equally reactive towards the host's own tissues (Sadd & Siva-Jothy 2006), positive covariation between investment in immune function (i.e. PO activity) and antioxidant defence is to be expected (Cornet *et al.* 2007). In insects, the main antioxidant enzymes neutralizing superoxide anions and hydrogen peroxide are superoxide dismutase (SOD) and catalase (CAT), respectively (Korsloot *et al.* 2004).

We examined the influence of autotomy of the caudal lamellae on investment in immune response (PO) and in antioxidant defence (SOD and CAT) in the larvae of the damselfly *Lestes viridis*, where lamellae autotomy occurs very frequently. Based on the resemblance to wounding, we expect a decreased investment in immune function after autotomy (Plaistow *et al.* 2003). Next, given the cytotoxicity of PO, we expect a positive covariation between investments in immune function and in antioxidant defence, both at the treatment level and the individual level.

2. MATERIAL AND METHODS

(a) *Sampling and experimental design*

Twigs containing eggs of *L. viridis* were brought into the laboratory where they were placed in large trays filled with pond water to a height of 1 cm. When the eggs hatch, the larvae fall into the water. After four weeks, larvae were placed individually in 100 ml cups filled with pond water. Throughout the larval stage, the larvae were kept at 21 ± 1°C and fed daily ad libitum with *Artemia nauplii*.

When larvae reached the final instar, we removed all three lamellae from one random half of the larvae by gently pulling them with two fingers until the animals autotomized these appendages at the specialized breaking joints (as in Stoks 1999; Stoks *et al.* 1999). The other half of the larvae underwent a sham operation without removing the lamellae. In all cups the water was replaced by oxygen saturated water. Twenty-four hours later, larvae were flash-frozen and stored at −80°C for analysis.

(b) *Response variables*

Larvae were weighted to the nearest 0.01 mg. Then, we spectrometrically quantified the kinetic activity of PO, total PO (including the proenzyme proPO), SOD and CAT following the existing protocols for the study species (see appendix in the electronic supplementary material). Samples were run in triplicate and their mean was taken as the value for the animal. The intraclass correlation coefficients for PO, SOD and CAT were 0.92, 0.89 and 0.30, respectively.

(c) *Statistical analyses*

As there was a strong correlation between the activity of PO and total PO ($r = 0.95$; $p < 0.0001$), and only the active PO causes cytotoxic molecules, we only report PO activity measurements. We estimated the effect of lamellae autotomy on the three physiological variables (PO, CAT and SOD) in a MANCOVA with protein content and mass as covariates. Sex and protein content were withdrawn from the model as these effects were not significant. We ran separate ANCOVAs for each response variable with lamellae autotomy and sex as independent variables. As covariates we initially included mass, protein content and the two other physiological variables. Interactions between lamellae autotomy and the covariates were never significant. Non-significant effects were withdrawn from the final model. To specifically evaluate covariation between PO, SOD and CAT at the individual level, we calculated for each of their pairwise combinations, the correlation coefficient between their residuals after accounting for lamellae status and the activity of the enzyme not included in the correlation.

3. RESULTS

Autotomy had an overall effect on the three physiological variables (MANCOVA, $F_{3,63} = 3.29$; $p = 0.026$, protein covariate: $F_{3,63} = 3.64$; $p = 0.017$). After autotomy, larvae had lower PO ($F_{1,87} = 6.36$; $p = 0.014$; SOD covariate: $F_{1,87} = 16.43$; $p < 0.001$; figure 1*a*) and SOD ($F_{1,87} = 7.15$; $p = 0.009$, PO covariate: $F_{1,87} = 16.43$; $p < 0.001$; figure 1*b*) activity. CAT activity was unaffected by autotomy ($F_{1,76} = 0.14$;

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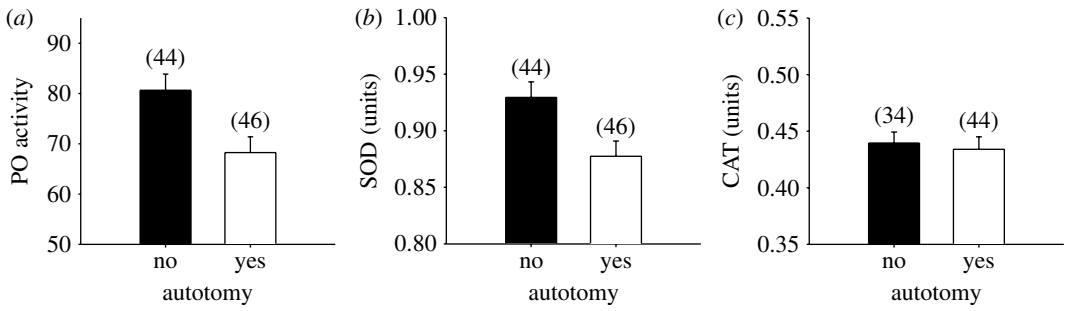

Figure 1. Mean (+1 s.e.) activities of (*a*) PO, (*b*) SOD and (*c*) CAT in *L. viridis* larvae in the function of lamellae autotomy. Numbers above bars represent sample sizes. For PO and SOD, least-squares means corrected for SOD and PO, respectively, are given.

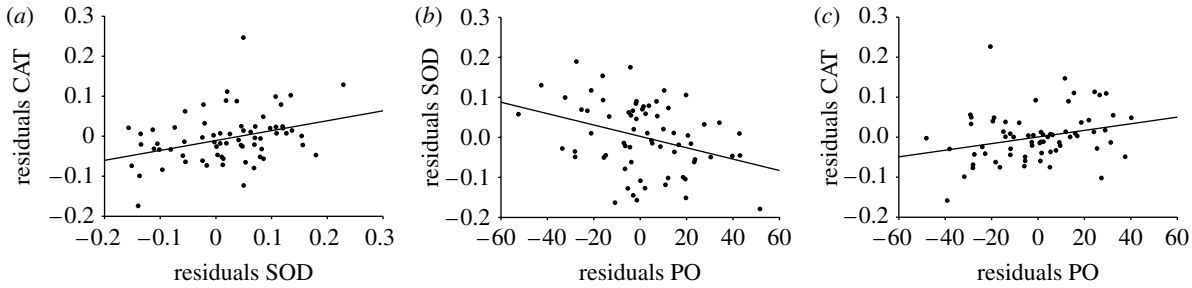

Figure 2. Relationships between residual activities of (*a*) SOD and CAT, (*b*) PO and SOD and (*c*) PO and CAT at the individual level.

$p=0.707$; figure 1*c*). The levels of both antioxidant enzymes correlated positively ($r=0.34$, $p=0.004$, $n=68$; figure 2*a*). PO activity was negatively correlated with SOD ($r=-0.34$, $p=0.001$; figure 2*b*) but positively with CAT ($r=0.27$, $p=0.026$; figure 2*c*) activity.

4. DISCUSSION

The PO reduction in the larvae with autotomized lamellae is the first demonstration of a reduced investment in immune function after autotomy. These results may, to some extent, be compared with the few studies on the effects of wounding on investment in immune function. An important difference is, however, that during autotomy no typical wound is created, instead, a body part is removed at a specific breaking joint, and in the case of lamellae autotomy by damselfly larvae a specialized sphincter directly closes to minimize loss of haemolymph and invasion by pathogens (MacNeill 1960). In line with this, there was no upregulation of PO 0.5 and 1 hour after autotomy, and already a downregulation after 2 hours (see appendix in the electronic supplementary material). Furthermore, the drop in PO (and SOD) levels could not simply be attributed to haemolymph loss as there was no drop in trehalase, another haemolymph enzyme not related to immune function (see appendix in the electronic supplementary material).

A possible reason for the PO reduction after autotomy is energy limitation and reallocation. Given its cytotoxicity, activated PO loses its activity rapidly, and to maintain constant levels there is a need for continuous production (Sugumaran 2002). Because this production is energetically costly (Siva-Jothy & Thompson 2002; Rolff *et al.* 2004), we hypothesize that the reduced energy status of larvae without lamellae may have caused the PO reduction. In line with this, former studies on *Lestes* showed lower foraging and growth rates after autotomy (Stoks 1999, 2001; Stoks *et al.* 1999). The decreased PO activity after 24 hours was not due to a decrease in overall protein synthesis rate as lamellae autotomy did not affect RNA/DNA ratios (see appendix in the electronic supplementary material). Presumably, the lowered energy status after autotomy caused a reallocation of energy at the expense of the investment in immune function. Similarly, Rigby & Jokela (2000) found in snails that an increased allocation to predator avoidance depressed the immune function. Potentially, more energy was shunted towards the induction of stress proteins as shown after tail autotomy in lizards (Merino *et al.* 2004). Such induction of stress proteins could be a result of increased predator stress (Slos & Stoks 2008) as after autotomy, damselfly larvae perceive a higher predation risk (Stoks 1998), or a result of lamellae loss acting as a stress *per se*.

Concurrent with the lowered levels of PO, autotomized larvae had lower SOD levels, which are costly to maintain (De Block & Stoks 2008). This decrease could therefore be a consequence of the lower energetic status and energy reallocation of the larvae and the lower need for antioxidant defence given the lowered metabolism after lamellae autotomy (Apodaca & Chapman 2004, but see Barja 2007). Additionally, given that the oxidation of phenols to quinones by PO generates reactive oxygen species (Nappi & Christensen 2005), the reduced PO levels may have reduced the need for high SOD levels. In line with our results, Mathew *et al.* (2007) showed in tiger shrimps that an experimentally induced PO reduction was associated with a decreased SOD

activity after 24 hours. Furthermore, Ballarin *et al.* (2002) showed in an ascidian that the cytotoxicity of PO could be suppressed by SOD. It is unclear why CAT levels did not show a similar decrease after autotomy. During starvation, CAT levels are lowered much less than SOD levels in *L. viridis* (De Block & Stoks 2008) indicating that CAT levels may be less costly to maintain. Alternatively, CAT may be relatively more abundant outside the haemolymph and therefore less important in preventing PO autocytotoxicity.

At the individual level, we found the expected positive covariation between both antioxidant enzymes and between PO and CAT. The latter pattern can be explained by the generation of reactive oxygen species by PO (Sugumaran 2002) so that individuals with higher PO may need a higher antioxidant defence. In the only other study of this question, Cornet *et al.* (2007) showed in *Gammarus pulex*, a positive correlation both at the population and at the individual level between levels of PO and an antioxidant (i.e. carotenoids). Intriguingly, PO and SOD covaried negatively at the individual level. One reason for this may be an energy-based trade-off between the production of both costly enzymes.

Autotomy is a widespread antipredator behaviour with, besides the obvious escape benefits, future costs including reduced mating success and survival (Fleming *et al.* 2007). These costs are typically attributed to reduced locomotory abilities. Our results point to a novel cost of autotomy in terms of a reduced investment in innate immune function, and therefore a decreased potential for future defence against pathogens. We hypothesize that, although this effect may be transient (see appendix in the electronic supplementary material), this window of reduced immune function may critically affect fitness. Future work should focus on the generality of our findings in other taxa. This reduced immune function associated with predator defence deserves further attention as a mechanism of how predators may make prey more vulnerable to parasites and as such, may play a role in indirect trait-mediated effects in food chains where predators and parasites typically co-occur.

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