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Opposite changes in the resistance to cold and desiccation, which occur during the development of the millipede *Polydesmus angustus* (Diplopoda: Polydesmidae)

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Abstract. Supercooling point (SCP), survival at low temperatures, rate of water loss in dry air at 20°C and survival under desiccating conditions of eggs of *Polydesmus angustus* (Diplopoda) were determined. The results were compared with those obtained previously for the eight post-embryonic stadia, to obtain an overview of the changes in resistance to cold and desiccation throughout the species' development. The SCP temperatures of egg batches ranged from –14.8 to –30.6°C and were significantly lower than those of the active stadia. Eggs were not affected by prolonged exposure to low temperature above 0°C and survived much better than active stadia when cooled to –6 and –10°C. This indicates that the cold hardiness of *P. angustus* is highest in the egg stage and decreases during development. On the other hand, the rate of water loss was significantly higher from eggs than from active stadia. When eggs were taken out of their protective nest, they lost water at the high rate of 7% min^{-1} in dry air. They also survived for a shorter time than active stadia at 76% RH and 20°C. The resistance to desiccation of *P. angustus* is lowest at the egg stage and increases during development. The results suggest that the life cycle of *P. angustus* may have responded to selection pressures other than cold and drought, and do not support the hypothesis that cold hardiness and resistance to desiccation are overlapping adaptations in terrestrial arthropods.

INTRODUCTION

The millipede *Polydesmus angustus* Latzel is common throughout the Atlantic climatic regions in N.W. Europe, where it lives in leaf litter and under the bark of decaying logs (Kime, 1990). Its life cycle is closely adjusted to seasonal changes, growth and reproduction being restricted to specific periods of the year, as in most temperate-zone arthropods (Tauber et al., 1986; Schaefer, 1987; Mocquard et al., 1989). It oviposits over a long period in spring-summer and adults diapause in early autumn, which precludes any reproduction in autumn-winter (David et al., 1999; unpublished data). Individuals born in the first part of the breeding season (May-August) overwinter as stadia V to VIII (adult) and reproduce when about 1 year old. Individuals born later (August-October) overwinter for the first time as stadia III to V, the second time as adults, and reproduce when nearly 2 years old. In neither case is winter spent in the egg stage or early juvenile stadia. In contrast, all life stages (eggs and the eight post-embryonic stadia) can be found in summer (Banerjee, 1973; David et al., 1993).

The survival of the active stadia of *P. angustus* at low temperatures (cold hardiness) and under desiccating conditions (desiccation resistance) was studied by David et al. (1996) and David & Vannier (2001). Overwintering stadia, which survive for several months at a few degrees above 0°C and tolerate short exposure to sub-zero temperatures above the supercooling point, can be regarded as chill-tolerant. However, all individuals die when they are frozen. The supercooling point (SCP) is therefore a fairly good indicator of cold hardiness in this millipede, which is not normally exposed to long periods of frost in the field. The SCP is relatively high (overall range from –10.6 to –2.2°C) and significantly increases from the first stadium to the adult stage. No significant seasonal variation was detected in stadia that occur in both summer and winter. Furthermore, this species has a very low desiccation resistance in summer. Although various stadia tolerate a 47% loss of body water, the rates of water loss are high, especially in juveniles, and the dehydration tolerance limit is quickly reached. In the absence of liquid water, first stadium juveniles survive for about 1 h at 76% RH and 20°C (4 mmHg saturation deficit).

This study presents further results on the SCP, survival at low temperatures, rate of water loss and desiccation resistance of *P. angustus* eggs. These traits were studied using the same methods as for post-embryonic stadia, so that the resistance to cold and desiccation of all life stages could be compared using the same criteria. The objective was to determine whether there is any relationship between the resistance to climatic stresses at the different life stages and the characteristics of the life cycle, in particular the restriction of reproduction to the spring-summer period.

In addition, the results are discussed from the viewpoint of the relationship between cold hardiness and desiccation resistance in terrestrial arthropods. Ring & Danks (1994) hypothesized that both traits may be determined by the

TABLE 1. Percentages of *P. angustus* eggs that hatched at 16°C after exposure of nests with eggs to different levels of cold. The results were compared with the survival of first stadium juveniles subjected to the same treatments in culture boxes. The significance levels for the differences are given in the text.

Treatment	Eggs		Juveniles	
	n	Hatching success (% per nest ± SE)	n	Survival (% per box ± SE)
3°C for 2 weeks	724 in 3 nests	100%	37 in 3 boxes	94 ± 6%
3°C for 4 weeks	523 in 2 nests	100%	34 in 3 boxes	94 ± 3%
3°C for 8 weeks	320 in 2 nests	98 ± 2%	39 in 3 boxes	90 ± 6%
3°C for 2 weeks and a short frost of –6°C	608 in 3 nests	98 ± 2%	42 in 3 boxes	61 ± 13%
3°C for 2 weeks and a short frost of –10°C	358 in 3 nests	76 ± 17%	47 in 3 boxes	6 ± 4%

same physiological mechanisms in insects, mainly the accumulation of low molecular weight substances like polyols and sugars. These substances are known to be effective colligative-type antifreezes that depress the SCP in many insect species (Zachariassen, 1985). On the other hand, increased levels of polyols and sugars were also found in insects at high temperatures, such as pupae of the moth *Operophtera brumata* during summer diapause (Ring & Danks, 1994). High concentrations of these substances lower the water vapour pressure of the haemolymph and reduce water losses (Bayley & Holmstrup, 1999). Hence the view that resistance to cold and desiccation are overlapping adaptations, which has received considerable attention in recent years (Block, 1996; Klok & Chown, 1998; Sømme, 1999; and references below).

MATERIAL AND METHODS

Egg production in the laboratory

Male-female pairs of *P. angustus* collected in winter at Brunoy, near Paris, were maintained in the laboratory at 16°C (14°C at night and 18°C during the day) under natural day lengths. They were kept in plastic boxes with a substrate of soil (*ca.* 1 cm) and fed on leaf litter supplemented with dry yeast. By the end of winter adult females had started to build nests, and layed some 100–300 eggs per nest. Polydesmid eggs are slightly sticky and form a coherent batch. The nest is a small cone of earthy faecal material, which is sealed by the female after egg-laying, and from which first stadium juveniles emerge by chewing a hole (Snider, 1981). A total of 30 egg batches, up to a week old, were used in the experiments.

Supercooling points

The SCP of eggs was determined at the standard cooling rate of 1°C min^{-1} using the same apparatus as used for the post-embryonic stadia (David et al., 1996). Batches of freshly laid eggs (n = 6) were taken out of the nests and put into Eppendorf tubes. A thermocouple was delicately inserted into the middle of each batch, to record the full range of SCPs as the temperature was decreased to –37°C.

Survival at low temperatures

Boxes containing one nest were kept for 2, 4 or 8 weeks in a chamber at 3°C, the mean temperature of the coldest month in the field. After this period of chilling, the nests were incubated for 3 weeks at 16°C to check the viability of the eggs. The percentages that hatched were determined by counting all first stadium juveniles in each box and the unhatched eggs inside the nests.

Other nests were exposed to colder conditions, by subjecting them to a frost in the middle of a 2-week period at 3°C. The chamber was cooled at a slower rate than for SCP determination (*ca.* 0.2–0.3°C min^{-1}) until the air temperature reached –6 or –10°C inside the boxes. The temperature was controlled using a probe inserted through the lid. The nests were held at the minimum temperature for 5–6 min and then returned to 3°C at a rate of 0.4°C min^{-1}. In total, the nests remained below 0°C for *ca.* 45 min when cooled to –6°C and for *ca.* 70 min when cooled to –10°C. The boxes were kept for a further week at 3°C and transferred to 16°C to check egg viability. The percentages that hatched were determined as above.

For comparative purposes, first stadium juveniles whose mean SCP is known to be –7.4 ± 0.5°C (David et al., 1996) were subjected to the same cold hardiness tests as the eggs (3 boxes per treatment; see Table 1 for sample sizes). The proportions of survivors were determined directly at the end of the cold periods.

Water loss rate and desiccation resistance

The rate of water loss of eggs was measured in dry air at a constant 20°C using the same apparatus as used for the post-embryonic stadia (David & Vannier, 2001). Batches of freshly laid eggs (n = 3) were taken out of the nests and immediately placed on the pan of a Cahn recording electrobalance (sensitivity 0.1 µg). The pan was suspended in a small chamber where the relative humidity was maintained at below 5% by silica gel (about 17 mmHg saturation deficit). The changes in egg-batch weight due to water loss were continuously recorded. The dry weight was obtained after drying at 60°C on the balance pan, and the rate of water loss was calculated for each batch as a percentage of the original water content lost per minute.

In order to compare the survival of eggs under desiccating conditions with that of the post-embryonic stadia, batches of freshly laid eggs (n = 4) were taken out of the nests, placed on filter paper in uncovered plastic pots (4.5 cm in diameter by 2.5 cm high) and kept for 1 h in a glass desiccator above a saturated solution of NaCl (76% RH at 20°C, i.e. 4 mmHg saturation deficit). Post-embryonic stadia are known to survive for 1 h in these conditions, with the exception of the first stadium juveniles, only 8% of which survive (David & Vannier, 2001). After 1 h in the desiccator, the filter paper supporting the eggs was moistened by putting wet cotton wool on it, the pots were sealed and incubated at 18°C for 3 weeks. The percentage of eggs that hatched in each pot was determined and the mean percentage was compared with that of control batches (n = 4) that were

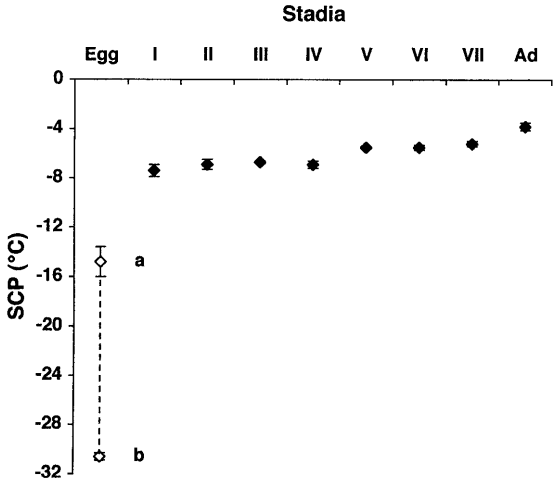

Fig. 1. Supercooling points (means ± SE) of *P. angustus* eggs (open symbols) and those of the eight post-embryonic stadia (closed symbols; from David et al., 1996). Egg batches (n = 6) were cooled and *a* and *b* correspond to the mean temperatures at which the first and the last eggs froze in the batches, respectively.

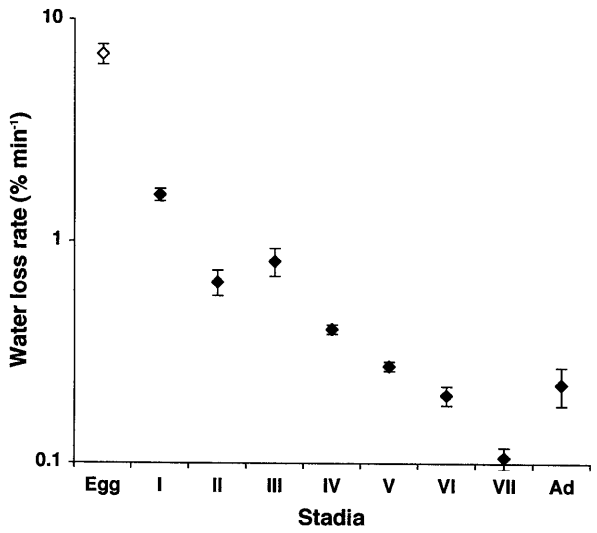

Fig. 2. Water loss rates in dry air at 20°C of *P. angustus* eggs (open symbol) and those of the eight post-embryonic stadia (closed symbols; from David & Vannier, 2001). The egg batches (n = 3) were desiccated out of their nests. The results, expressed as percentages of the original water content lost per minute (means ± SE), are plotted on a logarithmic scale.

taken out of the nests and incubated on moist filter paper without being subjected to desiccation.

RESULTS

Supercooling

The SCP of eggs was very variable, ranging from –10 to –31°C. It was not possible to calculate the mean for each batch because individual SCPs overlapped, but the highest and lowest SCP temperatures for each batch were recorded. On average, the first eggs froze at –14.8 ± 1.2°C (range –10.0 to –18.0°C) and the last eggs froze at –30.6 ± 0.3°C (range –29.1 to –31.3°C). The comparison with active stadia showed that SCP was much lower for eggs (Fig. 1). Even taking into account the highest SCP temperature for each egg batch (–14.8°C on average), there were highly significant differences between the life stages (ANOVA; $P < 0.001$) and multiple comparison tests using the Tukey-Kramer method showed that eggs started to freeze at a significantly lower temperature than the active stadia ($P < 0.01$).

Survival at low temperatures

The cold hardiness tests showed that most eggs remained viable after all the treatments (Table 1). The percentage hatching at 16°C ranged from 98 to 100% when nests were maintained for 2–8 weeks at 3°C or were cooled to –6°C. Although hatching success was lower when nests were cooled to –10°C, there was no significant difference between treatments. In fact, only one out of three nests had a low percentage hatch after exposure to –10°C, and the two others had scores ≥ 92%.

The tests conducted with first stadium juveniles under the same conditions showed that survival was high (90–94%) when millipedes were maintained for 2–8 weeks at 3°C (Table 1). However, in contrast to eggs, juveniles were affected by sub-zero temperatures. There was a highly significant difference between treatments (ANOVA; $P < 0.001$) and multiple comparison tests showed that survival was significantly lower at –10°C than at 3°C ($P < 0.01$), with an intermediate effect at –6°C. The comparison between eggs and juveniles after exposure to –6 and –10°C, showed that a significantly higher percentage of eggs survived in both cases (t-tests; $P < 0.05$).

Water loss in dry air

For each egg batch tested, the weight loss curve was linear for a few minutes, which indicated a constant rate of water loss. After that, the rate steadily decreased down to very low values, probably because of rapid exhaustion of the egg water reserves. Under the initial conditions, the mean rate of water loss was 7.0 ± 0.7% min^{-1}. The comparison with the mean rates of active stadia (Fig. 2) showed that there was a highly significant difference between the life stages (ANOVA; $P < 0.001$) and multiple comparison tests showed that the rate of water loss was significantly higher for eggs than for all active stadia ($P < 0.01$).

Desiccation resistance

Eggs kept for 1 h at a 4 mmHg saturation deficit collapsed and none developed after rehydration. On moist filter paper, desiccated eggs regained their initial shape but did not grow and turned yellow, unlike the controls that grew while remaining white. The percentage hatching was significantly lower for desiccated eggs (0%) than for controls (92 ± 3%) (t-test; $P < 0.001$). Compared to the survival of the least resistant active stadium under the same desiccating conditions – 8% survivors in stadium I (David & Vannier, 2001) – egg survival was even lower, although the difference was not significant.

DISCUSSION

A number of traits related to the resistance to cold and desiccation were compared for all the life stages of *P. angustus*. Survival at low temperatures above 0°C is the only trait that does not vary significantly between life stages – at least for 8 weeks of cold, since eggs and juveniles were not tested over longer periods. The other traits vary markedly during development and the study of eggs has confirmed the trends observed in the post-embryonic stadia (David et al., 1996; David & Vannier, 2001). The ability to supercool, which is greatest for eggs, decreases throughout development. Considerable variation in the individual SCP of eggs was recorded and the lowest values might be due to rapid dehydration of some unfrozen eggs in contact with the ice of other eggs (cf. Holmstrup & Zachariassen, 1996). Nevertheless, eggs start to freeze at –14.8°C on average, a much lower temperature than for active stadia. The results have also confirmed the obvious relationship between the SCP and the species' cold hardiness. Whilst the tolerance of chilling seems to be similar at all life stages, survival after short-term exposures to sub-zero temperatures varies as a function of SCP. Thus, most eggs survive at –10°C, most juveniles with a mean SCP of –7.4°C are killed between –6 and –10°C, and overwintering stadia with higher SCPs do not survive when cooled at –6°C (David et al., 1996). These results indicate that cold hardiness is highest for eggs and decreases during development. On the other hand, the rate of water loss is much higher for eggs than for active stadia, and eggs extracted from a nest and kept under desiccating conditions survive for a very short time. Desiccation resistance is lowest for eggs and increases during development. The only exception is aestivating sub-adults of stadium VII, which show a significantly greater desiccation resistance than adults (David & Vannier, 2001).

Similar trends for changes in the resistance to cold and desiccation have been reported in other arthropod species and can be explained in terms of changes in body size. The SCP temperature of water increases with the volume of liquid, and this is true for arthropods provided that specimens of the same species in the same feeding condition are compared (Salt, 1966; Lee, 1991). The weight-specific rate of water loss decreases with decrease in surface area: Body weight ratio, i.e. when body size increases (Edney, 1977; Hadley, 1994). In addition, Gromysz-Kalkowska & Stojalowska (1973) showed that the weight-specific respiration rate of *Polydesmus complanatus* – a close relative of *P. angustus* – decreased regularly from the first stadium to the adult stage. As the respiratory water loss is the preponderant water loss in non-teneral polydesmoid millipedes (Stewart & Woodring, 1973), the rate of water loss must decrease during development, other things being equal.

These changes in resistance to climatic stresses during growth and development are not necessarily adaptive. For example, the low SCP of *P. angustus* eggs is of little adaptive significance in the population studied, since the species never overwinters as eggs. Egg-laying only takes place during the warmer half of the year, from late March to late September with a peak in June-July – a period when eggs have no need for much protection against frost. Many other examples of low SCPs have been reported for arthropod eggs laid in spring-summer (Block et al., 1990; David & Vannier, 1996; Vernon et al., 1997). On the other hand, the significantly lower rate of water loss in stadium VII individuals in aestivation seems to be adaptive for *P. angustus* (David & Vannier, 2001).

Considering the ecophysiological characteristics of the life stages, the seasonal timing of reproduction in *P. angustus* may seem paradoxical. The species has evolved diapause that precludes overwintering in stages capable of resisting cold (eggs and juveniles) and reproduction occurs when the risks of desiccation are greatest. During the summer, even assuming that eggs are well protected from desiccation in their closed nest, first stadium juveniles that leave the nest may be very exposed. This paradox can only be explained by assuming that if eggs and juveniles were to overwinter, they would incur major costs despite their ability to resist cold. Several hypotheses can be considered. (1) It is possible that eggs and first stadium juveniles are unable to survive chilling for more than 8 weeks, in contrast to larger stadia. This hypothesis, however, is not easy to test for eggs, because in long-term exposures it may be difficult to distinguish between mortality caused by cold and that caused by fungi and micro-organisms in the nests (hypothesis 3 below). (2) Even if eggs and juveniles survive low temperatures, overwintering could jeopardize their subsequent development in spring. After 8 weeks of cold, eggs hatch normally and first stadium juveniles moult into stadium II, but the effects of a complete winter should be confirmed before dismissing the hypothesis of a delayed effect on development. (3) Another hypothesis is that the avoidance of reproduction before winter could be explained in terms of predation risk. Embryonic development, which lasts *ca.* 3 weeks at 16°C, would be retarded for several months at winter temperatures, as Snider (1981) has shown in another *Polydesmus* species. Over such long periods, overwintering eggs could risk attack by fungi and other predators, as in many insect species (Leather et al., 1993). Aphids, for example, overwinter as eggs in cold regions but as less cold hardy active stages under milder climates, and it has been suggested that this could be related to predation on the eggs (Leather et al., 1993; Strathdee et al., 1995). The role of predation in the evolution of the life cycle of *P. angustus* remains speculative but, unless an adverse effect of cold on eggs and juveniles has been overlooked, it seems that the life cycle cannot be interpreted solely in terms of selective pressures resulting from cold and drought.

Finally, the results are relevant to any discussion of the relationship between cold hardiness and desiccation resistance in terrestrial arthropods. The overlapping adaptations hypothesis (Ring & Danks, 1994) states that the life stages of an arthropod species that have evolved increased resistance to one stress (cold or drought) are expected to show resistance to the other one as well.

Although relatively few studies have compared both cold and desiccation resistance within a species, some have revealed an association between the two traits. For example, during the post-embryonic development of the sub-Antarctic wingless fly *Anatalanta aptera*, the pupal stage is the most desiccation-resistant and shows the lowest SCPs (Vannier, 1985; Vernon & Vannier, 1996). In contrast, there is no association between desiccation resistance (high) and cold hardiness (low) during the summer diapause of the Mediterranean tiger moth *Cymbalophora pudica* (Kostal et al., 1998). Similarly, in adults of the oribatid mite *Phauloppia* spp. whose resistance to climatic stresses varies seasonally, the lowest SCPs occur in winter and correspond to an increased osmolality, and desiccation resistance is lowest in this season (Sjursen & Sømme, 2000).

The *P. angustus* results do not support for this species the hypothesis that cold hardiness and desiccation resistance are linked. In aestivating sub-adults of stadium VII, it is not known whether the increase in desiccation resistance is reflected in the SCP, because the SCP of stadium VII was only determined in winter and not during summer dormancy. However, the data collected during the same seasons, i.e. in spring for eggs and summer for stadia I-V, show that millipedes lose their ability to supercool as they gain resistance to desiccation. The two traits vary substantially from the egg stage to stadium V but, clearly, they are not associated.

It is likely that the contradictory reports on the association between traits result from the diversity of factors that influence either cold hardiness (e.g. ice nucleators, antifreeze proteins) or desiccation resistance (e.g. cuticular permeability) or even both (like solute concentration and body size). These factors may combine and interact in various ways, so that cold hardiness and desiccation resistance appear to be dependent in certain species and not in others. As discussed by Denlinger & Lee (1998) in another context, the two traits may be associated or not, and when associated, the relationship may be coincidental or linked. All these possibilities probably occur in terrestrial arthropods.

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