Functional Ecology 2004
18, 000–000

Phenotypic plasticity in pigmentation in *Daphnia* induced by UV radiation and fish kairomones

R. TOLLRIAN† and C. HEIBL

Section Evolutionary Ecology, Department Biology II, Ludwig-Maximilians Universität München, Karlstr. 23–25, 80333 München, Germany

Summary

1. Planktonic organisms are exposed to harmful ultraviolet (UV) radiation. Pigmentation offers protection but at the same time increases visibility, and therefore vulnerability, to visually orienting predators such as fish. As an adaptation against fish predation, zooplankton should be transparent, though this would leave them less protected against UV radiation. Thus both adaptations would appear to be mutually exclusive. However, phenotypic plasticity in pigmentation could allow flexible adaptation to both environmental situations.
2. We tested the hypothesis that *Daphnia* should be able to change their level of pigmentation in response to fish kairomone and/or UV radiation using four species of *Daphnia*.
3. *Daphnia hyalina* Leydig increased pigmentation under UV radiation and *D. pulex* Leydig reduced pigmentation in the fish kairomone treatment. Both species live in habitats with variable UV and fish impact.
4. *Daphnia cucullata* Sars and *D. middendorffiana* Fischer showed no reaction, probably because of their extreme adaptations: *D. middendorffiana* is strongly pigmented and seems to be adapted to high UV-B impact and an absence of fish in its arctic habitat. In contrast, *D. cucullata* has evolved in coexistence with fish. It can afford being nearly transparent because it lives in eutrophic lakes where UV-B is not relevant.
5. Our data on four species suggest that plasticity in pigmentation might be common in *Daphnia* adapted to environments with contrasting or variable selection pressures.

1

Key-words: Cladocera

Functional Ecology (2004) **18**, 000–000

Introduction

Zooplankton in nature is endangered by several biotic and abiotic factors, which sometimes necessitate mutually exclusive adaptations. For example, daphnids are confronted with predation by visually hunting predators. In this context the evolution of nearly transparent carapaces and of a small body size can be explained as camouflage (e.g. Kerfoot & Lynch 1987). On the other hand, UV radiation can also have an important impact, especially in shallow water bodies or near the surface in deeper pools and lakes (Calkins & Thordadottir 1980; Williamson *et al*. 1994). UV-B is the most dangerous part of the spectrum as it can lead to DNA-damage and consequently lethal and sub-lethal effects (e.g. Siebeck & Böhm 1994; Williamson *et al*. 1994; Zagarese *et al*. 1994). Melanin pigmentation as well as carotenoids are known to increase the UV tolerance of planktonic species (Hairston 1976; Hebert & Emery 1990; Rhode, Pawlowski & Tollrian 2001). Some species of *Daphnia* develop pigmentation to minimize UV-B hazards, but at the same time are likely to pay a cost in the presence of predators by being more visible (Luecke & O'Brien 1981; Hessen *et al*. 1999; Hansson 2000; Johnsen & Widder 2001).

Several cladoceran species or clones live in environments where they can be exposed to both threats. Behavioural adaptations such as diurnal vertical migration (DVM) are a potential solution to this problem in deeper lakes. Indeed, DVM has been shown to be inducible by both chemical cues released by fish (e.g. Dodson 1988; DeMeester *et al*. 1999) and UV-B radiation (Leech & Williamson 2001; Rhode, Pawlowski & 2
Tollrian 2001). However, DVM carries severe costs if daphnids have to migrate into cooler strata, because development times are temperature dependent (Loose & Dawidowicz 1994).

An alternative solution to this conflict would be offered if pigmentation were phenotypically plastic and inducible in response to fish cues and/or UV radiation. Such an adaptation would broaden the

---

†Author to whom correspondence should be addressed. E-mail: tollrian@lmu.de

FEC_870 | Pages: 6 | Proofreader: Chen Xiao Ming

fundamental niche. In accordance with this hypothesis the induction of pigmentation has been shown in copepods, another group of pelagic crustaceans (Hansson 2000). Phenotypic plasticity in defences is common in many organisms (Tollrian & Harvell 1999). For *Daphnia*, chemical sensitivity to fish kairomones (Dodson 1988; reviewed in Tollrian & Dodson 1999) and UV receptors (Smith & Macagno 1990), as well as the ability to see UV radiation (Merker 1930) have been shown. Thus the 'infrastructure' for risk assessment exists. We therefore tested in this study the hypothesis that species of the genus *Daphnia* might possess the ability to change their pigmentation in response to the presence of fish kairomones and/or UV radiation.

Materials and methods

EXPERIMENTAL ANIMALS

For this experiment we selected clones of four species of *Daphnia*, which differ in their typical environments and probably have evolved different adaptations. *Daphnia cucullata* is a fragile and nearly transparent species which is adapted to coexistence with fish (Hrbácek 1962). This species occurs mainly in eutrophic lakes, where UV-B is readily absorbed at the surface. Therefore, only a moderate degree of pigmentation and a stronger adaptation against fish predation should be expected. The clone used here stems from Lake Thaler See (Germany). *Daphnia hyalina* was isolated from Lake Konstanz (Germany). It is larger than *D. cucullata*, but is also transparent and co-occurs with fish. Because it resides in oligo- to mesotrophic lakes, where a UV impact exists in the epilimnion, a reaction to both factors is possible. Also in *D. pulex* a response to both factors is possible. *Daphnia pulex* is larger than the aforementioned species and thus is more vulnerable to fish predation, which can be relevant in its native range. On the other hand, it is possible that UV radiation cannot be avoided in shallower ponds. The clone used in our study had been isolated from a pond in Churchill in arctic Canada. *Daphnia middendorffiana*, strongly pigmented and the biggest of the species tested, stems from Uummaannaq Island near Greenland (the last two clones were kindly provided by L. Weider). This high arctic species inhabits shallow ponds where there is no predation risk by fish but where there is a high impact of UV-B radiation. This high-radiation impact is due to the lack of significant levels of dissolved organic carbon (DOC) compounds in the water, which would absorb parts of the UV radiation, as well as the shallow depth. However, because the intensity of UV-B changes seasonally we potentially could find plasticity in pigmentation. All clones had been kept in the laboratory for some generations.

DESCRIPTION OF TREATMENTS

In this study we aimed at determining whether there are responses in pigmentation to fish cues or UV radiation. We did not attempt to pinpoint response thresholds. We therefore applied relatively high doses of fish kairomone and UV to the experimental groups. We tested the effects of UV radiation and fish kairomones against a control group in parallel. The daphnids were kept in little glass bowls each containing 120 ml of artificial medium (SSS medium, Jeschke & Tollrian 2000). Two egg-carrying daphnids were placed in each bowl. The offspring were collected and distributed equally over every treatment and replicate to get equal densities in the bowls. The medium was changed daily to ensure a constant concentration of the kairomone and to keep the medium clean of organic substances. The daphnids were fed with *Scenedesmus obliquus* (Turpin) Kützing *ad libitum* (1·5 mg C l^{-1}). 3

The control treatment was irradiated with three fluorescence tubes, 58 W each, yielding a total of 178 W. To ensure equal illumination of the experimental vessels, the bowls were aligned in a double row with one of the fluorescence tubes installed about 30 cm over the bowls and the other two about 50 cm overhead.

The kairomone treatment was essentially the same except for the medium, which contained kairomones. The kairomones had been obtained from 60 small Minnows (*Phoxinux phoxinus* Linnaeus, 2 cm length) that had been kept in an aerated beaker in 3 l medium for 8 h. The resulting solution was diluted in order to achieve a concentration of five small fishes per litre per day in the medium. In the UV treatment one of the three fluorescence tubes was replaced with a UV tube (Philips TL40 W/12: 275–365 nm, max. 315 nm). To 4
filter the UV-C, this tube was covered with acetate foil. We selected a slightly lower visible-light intensity in the UV treatment to exclude the possibility that the visible light could have caused any induction of pigmentation. The fluorescence tubes were switched on daily from 5.00 am to 9.00 pm (16 h). The UV tube was switched on from 7.00 to 9.00 am because prior tests with *D. pulex* had indicated no lethal effect for a 2-hour irradiation.

MEASUREMENTS AND ANALYSIS

Pictures were taken of every experimental animal at the age of 6 days (before they reached maturity) using a digital camera (Nikon Coolpix 990) mounted 5
on a stereomicroscope. To be able to compare the pigmentation without artefacts, illumination, aperture and exposure were kept constant. For measurement of body size we calibrated the images by photographing a micrometer under the same conditions.

We measured the intensity of pigmentation in every individual as the grey-scale values of the pixels in the photographs in two transects. The mean of all the grey values in the first transect represents pigmentation of the individual in the neck region and is therefore referred to as '*neck*'. In a similar manner, we calculated a variable '*back*' by measuring a transect inside the empty brood pouch. As carapace thickness probably increases with increasing body size and pigmentation is possibly correlated with carapace thickness, the body size of *Daphnia* could potentially affect the intensity of pigmentation. To test if there is any relation between the size of individuals and the degree of pigmentation we also measured the *body size* of all individuals from the compound eye to the base of the tail spine. All measurements in digital images were carried out with the software AnalySIS 2·11 (Soft Imaging Systems, Münster, Germany).

Statistical tests were performed with SPSS version 10·1 (SPSS Inc., Cary, NC). Because data were normally distributed and had equal variances, we calculated one-way ANOVAS for each species to find differences between treatments in the variables neck, back and body size. We also calculated minimum significant differences (MSD, for $\alpha = 0·05$) with Tukey–Kramer HSD tests for pairwise comparisons (Sokal & Rohlf 1995). To evaluate the effect of body size on pigmentation we conducted linear regressions for species and treatments.

Results

GROWTH

To check for size differences, which could have influenced our pigmentation results, we compared the body size of individuals at the age when pigmentation was measured. Only with *D. cucullata* (being smaller in the fish treatment) did the growth rates in the kairomone treatment differ from the control (Table 1). The UV treatment had a negative impact on growth rates and survival. In *D. pulex* and *D. middendorffiana* the UV-exposed animals were smaller than in the control and fish treatments (Table 2). The greatest impact of the UV radiation, however, occurred in the small *D. cucullata*. In this treatment all individuals died and thus could not be used for the analysis of UV effects. Growth in *D. hyalina* was not affected by UV radiation (Table 1).

Table 1. Results of ANOVAS comparing body size among treatments. In *D. cucullata* only fish and control treatments could be compared. Significant *P*-values are printed in bold

Species		Sum of squares	d.f.	Mean square	*F*	*P*
D. cucullata	Between groups	0·0346	1	0·0346	29·225	**<0·001**
	Within groups	0·0450	38	0·0012		
D. hyalina	Between groups	0·1380	2	0·0690	1·6019	0·208
	Within groups	3·4877	81	0·0471		
D. middendorffiana	Between groups	0·7053	2	0·3527	36·8852	**<0·001**
	Within groups	0·2964	31	0·0096		
D. pulex	Between groups	0·8070	2	0·4035	53·8091	**<0·001**
	Within groups	1·2148	162	0·0075		

Table 2. Results of Tukey–Kramer HSD tests comparing single pairs of means in body size for *D. middendorffiana* and *D. pulex*. Positive significance values are printed in bold and show pairs of means that are significantly different (difference between treatments is larger than the minimum significant difference, MSD; Sokal & Rohlf 1995) ($\alpha = 0·05$)

Species	Treatment (i)	Treatment (j)	Dif = Mean(i) – Mean(j)	Significance
D. middendorffiana	Control	Kairomone	−0·048	−0·062
	Control	UV	0·262	**0·155**
	Kairomone	UV	0·310	0·215
D. pulex	Control	Kairomone	0·013	−0·023
	Control	UV	0·174	**0·131**
Kairomone	UV	0·161	0·119	

BODY SIZE AND PIGMENTATION

The only species that showed a significant influence of body size on pigmentation was *D. pulex*. In this clone the linear regression of '*neck*' as well as of '*back*' on body size were highly significant in the kairomone treatment ($P < 0·001$), indicating that bigger animals in this treatment were also more strongly pigmented. However, we did not detect differences in body size in *D. pulex* between the kairomone treatment and control (Table 2).

PIGMENTATION

All data were normally distributed. All variances were homogeneous. We found significant plasticity in pigmentation in both induction treatments (Fig. 1). *Daphnia hyalina* responded to UV radiation by increasing its pigmentation. Differences in means were significant in '*neck*' as well as in '*back*' (Tables 3 and 4). In the kairomone treatment we found that *D. pulex* responded by reducing pigmentation. The differences in '*neck*' and '*back*' to control and UV treatments proved to be highly significant (Tables 3 and 4).

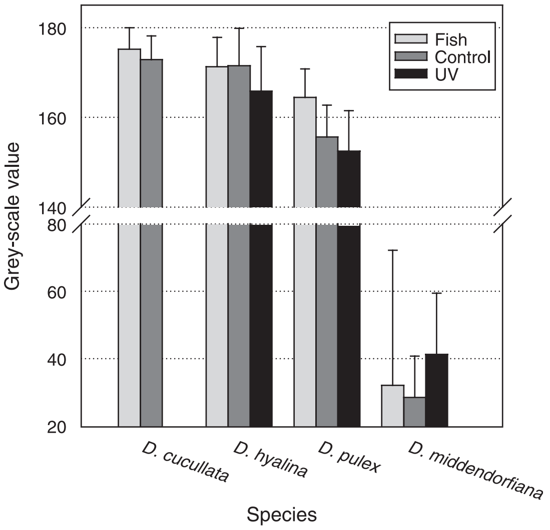

Fig. 1. Pigmentation in four *Daphnia* species in the '*neck*' region in response to fish kairomone, control and UV treatment (means ± SD). Pigmentation is shown with grey-scale values. Lower values mean stronger pigmentation. Pigmentation increased significantly in *D. hyalina* in the UV treatment and decreased significantly in *D. pulex* in the fish kairomone treatment. *Daphnia cucullata* is the most transparent species; *D. middendorffiana* is permanently strongly pigmented.

Table 3. Results of ANOVA comparing differences in pigmentation intensity for variables ‘*neck*’ and ‘*back*’ among treatments. Because of its strong pigmentation ‘*back*’ could not be measured in *D. middendorffiana*. Significant *P*-values are printed in bold

Species			Sum of squares	d.f.	Mean square	*F*	*P*
D. cucullata	Neck	Between groups	51·162	1	51·162	2·049	0·160
		Within groups	948·868	38	24·970		
	Back	Between groups	0·167	1	0·167	0·008	0·927
		Within groups	746·756	38	1·651		
D. hyalina	Neck	Between groups	452·707	2	226·353	3·504	**0·035**
		Within groups	5232·618	81	64·600		
	Back	Between groups	290·084	2	145·042	3·862	0·026
		Within groups	2703·997	72	37·556		
D. middendorffiana	Neck	Between groups	994·723	2	497·762	0·669	0·520
		Within groups	23079·582	31	744·503		
D. pulex	Neck	Between groups	2917·710	2	1458·86	30·844	**<0·001**
		Within groups	7662·357	162	47·30		
	Back	Between groups	1376·788	2	688·394	16·332	**<0·001**
		Within groups	6828·122	162	42·149		

No significant effect of the kairomone treatment was found in *D. cucullata* (Table 3). Similarly, the *D. middendorffiana* clone showed no plasticity. Neither individuals from the kairomone nor individuals from the UV treatment differed significantly from the control (Table 3).

Within all species and treatments we obtained the same responses in both independently measured traits ‘*neck*’ and ‘*back*’. The consistency of this double-check emphasizes the suitability and applicability of our measurement parameters.

Discussion

With this experiment we aimed to search for phenotypic plasticity in body pigmentation of *Daphnia.* As a general tendency (though with clonal differences), we expected to find a reduction of pigmentation in the presence of fish kairomones and an increase in pigmentation caused by UV radiation. Both types of plasticity would broaden the niche of the organism in heterogeneous environments, as they provide a greater flexibility to adapt to changes in irradiation and predation pressure. For example, early in the year, when the water is clear and much hazardous UV-B radiation penetrates the water, the generations of *Daphnia* would need to be pigmented in order to be better protected. Later in the year, the abundance of young fish increases predation risk and daphnids should reduce their pigmentation and become more transparent.

Table 4. Results of Tukey–Kramer HSD tests comparing single pairs of means in variables ‘*neck*’ and ‘*back*’ for *D. hyalina* and *D. pulex*. Positive significance values are printed in bold and show pairs of means that are significantly different (difference between treatments is larger than the minimum significant difference, MSD; Sokal & Rohlf 1995) (α = 0·05)

Species	Variable	Treatment (i)	Treatment (j)	Dif = Mean(i) – Mean(j)	Significance
D. hyalina	Neck	Control	Kairomone	0·221	−4·545
		Control	UV	5·660	**0·068**
		Kairomone	UV	5·439	−0·058
	Back	Control	Kairomone	1·810	−2·149
		Control	UV	5·313	**0·698**
		Kairomone	UV	3·503	−0·772
D. pulex	Neck	Control	Kairomone	−8·812	**5·923**
		Control	UV	−0·839	−2·584
		Kairomone	UV	7·973	**4·667**
	Back	Control	Kairomone	−5·873	3·146
		Control	UV	−0·072	−3·159
		Kairomone	UV	5·800	2·679

Our study provides two results that support our hypothesis and can be interpreted as adaptive plasticity. First, *D. hyalina* showed significantly increased pigmentation under influence of UV radiation. As it coexists with fish and is exposed to UV radiation, this seems to be a relevant trait. Later in the year, when fish predation risk increases, this species performs DVM which might explain the lack of a response to the fish kairomone. This significant increased pigmentation could not have been caused by differences in body size, because such differences were not significant (Table 1) and there was no significant correlation between pigmentation and body size.

Second, the pigmentation of *D. pulex* was significantly reduced in the fish-kairomone treatment in comparison to the control and UV treatments. *Daphnia pulex* also showed a stronger pigmentation as compared to the two Central European species *D. cucullata* and *D. hyalina.* This renders it more visible to fish and hence we should expect phenotypic plasticity in pigmentation to be advantageous. Again our result could not have been caused by body-size effects because body sizes were not significantly different between kairomone treatments and the controls (Table 2). Nevertheless, we found a significant correlation between pigmentation and body size in the kairomone treatment with larger animals being more pigmented. Thus, the kairomone possibly has a stronger influence on the pigmentation of smaller animals.

The pigmentation of *D. middendorffiana* did not change in either the kairomone or in the UV treatment.

This strongly pigmented species lives in shallow ponds in the arctic region where it is exposed to heavy doses of UV-B radiation. Our data suggest that this species evolved a sufficiently protective level of pigmentation. This is supported by the fact that individuals survived very well in the UV treatment. The lack of a response against fish kairomones is to be expected because of the absence of these predators in the species' native habitats.

Daphnia cucullata represents the other extreme of possible adaptations. Individuals are transparent and relatively small, and their coevolution with fish is quite obvious (Hrbácek 1962). The individuals in the kairomone treatment did not respond by decreasing pigmentation, which indicates that greater transparency is either not reasonable or not possible. The total loss of all individuals in the UV treatment indicates that this species is extremely specialized in its adaptation. *Daphnia cucullata* occurs exclusively in mesotrophic to eutrophic lakes where high concentrations of DOC absorb the harmful UV-B within the first few centimetres of the water column.

Our data show that phenotypically plastic responses in body pigmentation can indeed be observed in some *Daphnia* species in response to fish kairomones and UV radiation. Inducible pigmentation could confer evolutionary advantages under heterogeneous environments, especially if it is combined with other UV-tolerance mechanisms (Malloy *et al*. 1997) such as photoenzymatic repair (Sutherland 1981) or the excision repair system (Sancar 1994). This is because it simultaneously allows for the avoidance of predation and the negative effects of UV radiation. Perhaps species that live in environments where both factors of fish predation and UV radiation are relevant and variable may even show reciprocal responses to these factors. Other clones of *D. pulex* could potentially be good candidates to respond to both cues. However, the local adaptations of the clones will be of critical importance. It may be expected that, in particular, induced transparency should be a common defence against fish predation in temperate lakes. Further studies in this area are clearly needed and will provide valuable insights into reaction thresholds, mechanisms and the micro-evolution and local adaptations of different clones and species.

Acknowledgements

We thank M. Kredler for support during experiments. C. Laforsch, S. Stevens and two anonymous reviewers provided valuable comments. Our study was funded by the Deutsche Forschungsgemeinschaft (DFG).

References

Calkins, J. & Thordadottir, T. (1980) The ecological significance of solar UV radiation on aquatic organisms. *Nature* **283**, 563–566.

De Meester, L., Dawidowicz, P., van Gool, E. & Loose, C.J. (1999) Ecology and evolution of predator-induced behavior of zooplankton: depth selection behavior and diel vertical migration. *The Ecology and Evolution of Inducible Defenses* (eds R. Tollrian & C.D. Harvell), pp. 160–176. Princeton University Press, Princeton, NJ.

Dodson, S.I. (1988) The ecological role of chemical stimuli for the zooplankton – predator-avoidance behavior in *Daphnia*. *Limnology and Oceanography* **33**, 1431–1439.

Hairston, N.G. Jr (1976) Photoprotection by carotenoid pigments in the copepod *Diaptomus nevadensis*. *Proceedings of the National Academy of Sciences USA* **73**, 971–974.

Hansson, L.A. (2000) Induced pigmentation in zooplankton: a trade-off between threats from predation and ultraviolet radiation. *Proceedings of the Royal Society London B* **267**, 2327–2331.

Hebert, P. & Emery, J. (1990) The adaptive significance of cuticular pigmentation in *Daphnia*. *Functional Ecology* **4**, 703–710.

Hessen, D.O., Borgeraas, J., Kessler, K. & Refseth, U.H. (1999) UV-B susceptibility and photoprotection of Arctic *Daphnia* morphotypes. *Polar Research* **18**, 345–352.

Hrbácek, J. (1962) Species composition and the amount of zooplankton in relation to fish stock. *Rozpravy Ceskoslovenske Akademie Ved, Rada Matematickych a Prirodnich Ved* **72**, 1–117.

Jeschke, J.M. & Tollrian, R. (2000) Density-dependent effects of prey defences. *Oecologia* **123**, 391–396.

Johnsen, S. & Widder, E.A. (2001) Ultraviolet absorption in transparent zooplankton and its implications for depth distribution and visual predation. *Marine Biology* **138**, 717–730.

Kerfoot, W.C. & Lynch, M. (1987) Branchiopod communities: associations with plankivorous fish in space and time. *Predation, Direct and Indirect Impacts on Aquatic Communities* (eds W.C. Kerfoot & A. Sih), pp. 367–378. University Press of New England, Hanover.

Leech, D.M. & Williamson, C.E. (2001) *In situ* exposure to ultraviolet radiation alters the depth distribution of *Daphnia*. *Limnology and Oceanography* **46**, 416–420.

Loose, C.J. & Dawidowicz, P. (1994) Trade-offs in diel vertical migration by zooplankton: the cost of predator avoidance. *Ecology* **75**, 2255–2263.

Luecke, C. & O'Brien, W.J. (1981) Phototoxicity and fish predation: selective factors in color morphs in *Heterocope*. *Limnology and Oceanography* **26**, 454–460.

Malloy, K.D., Holman, M.A., Mitchell, D. & Detrich, H.W. III (1997) Solar UVB-induced DNA damage and photoenzymatic DNA repair in Antarctic zooplankton. *Proceedings of the National Academy of Sciences USA* **94**, 1258–1263.

Merker, E. (1930) Sehen die Daphnien ultraviolettes Licht? *Zoologische Jahrbücher Abteilung Allgemeine Zoologie und Physiologie der Tiere* **48**, 277–348.

Rhode, S.C., Pawlowski, M. & Tollrian, R. (2001) The impact of ultraviolet radiation on the vertical distribution of zooplankton of the genus *Daphnia*. *Nature* **412**, 69–72.

Sancar, A. (1994) Mechanisms of DNA excision repair. *Science* **266**, 1954–1956.

Siebeck, O. & Böhm, U. (1994) Challenges for an appraisal of UV-B effects upon planktonic crustaceans under natural radiation conditions with a non-migrating (*Daphnia pulex obtusa*) and a migrating cladoceran (*Daphnia galeata*). *Archiv für Hydrobiologie Beiheft* **43**, 197–206.

Smith, K.C. & Macagno, E.R. (1990) UV photoreceptors in the compound eye of *Daphnia magna* (Crustacea Branchiopoda) a fourth spectral class in single ommatidia. *Journal of Comparative Physiology A* **166**, 597–606.

Sokal, R.R. & Rohlf, F.J. (1995) *Biometry*. Freeman & Co, New York.

Sutherland, B.M. (1981) Photoreactivation. *Bioscience* **31**, 439–444.

Tollrian, R. & Dodson, S.I. (1999) Inducible defenses in Cladocera. *The Ecology and Evolution of Inducible Defenses* (eds R. Tollrian & C.D. Harvell), pp. 177–202. Princeton University Press, Princeton, NJ.

Tollrian, R. & Harvell, C.D. (1999) *The Ecology and Evolution of Inducible Defenses*. Princeton University Press, Princeton, NJ.

Williamson, C.E., Zagarese, H.E., Schultze, P.C., Hargreaves, B.R. & Seva, J. (1994) The impact of short-term exposure to UV-B radiation on zooplankton communities in north temperate lakes. *Journal of Plankton Research* **16**, 205–218.

Zagarese, H., Williamson, C.E., Mislivets, M. & Orr, P. (1994) The vulnerability of *Daphnia* to UV-B radiation in the Northeastern United States. *Archiv für Hydrobiologie Beiheft* **43**, 207–216.

Received 14 August 2003; revised 20 January 2004; accepted 18 February 2004

Author Query Form

Journal: Functional Ecology

Article: fec_870.fm

Dear Author,

During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.

Many thanks for your assistance.

No.	Query	Remarks
1	do you wish to add more keywords that are not in the title of the article?	
2	**Leech & Williamson 2000** has been changed to **Leech & Williamson 2001** to match the list	
3	Is the text OK: C stands for coulomb	
4	Please supply company address for **Philips TL40 W/12**	
5	Please supply company address for **Nikon Coolpix 990**	
6	Please change this reference to match the text –De Meester or DeMeester?	

MARKED PROOF

Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

Instruction to printer	*Textual mark*	*Marginal mark*
Leave unchanged	• • • under matter to remain	Stet
Insert in text the matter indicated in the margin	⋏	New matter followed by ⋏
Delete	⊢⊣ through matter to be deleted	δ
Delete and close up	⊖ through matter to be deleted	⁀δ‿
Substitute character or substitute part of one or more word(s)	/ through letter or ⊢⊣ through word	New letter or new word
Change to italics	— under matter to be changed	⊔⊔
Change to capitals	≡ under matter to be changed	≡
Change to small capitals	= under matter to be changed	=
Change to bold type	〰 under matter to be changed	〰
Change to bold italic	≂ under matter to be changed	⊔⊔ 〰
Change to lower case	Encircle matter to be changed	≢
Change italic to upright type	(As above)	⊔̸
Insert 'superior' character	/ through character or ⋏ where required	γ under character e.g. $\overset{2}{\gamma}$
Insert 'inferior' character	(As above)	⋏ over character e.g. $\underset{2}{⋏}$
Insert full stop	(As above)	⊙
Insert comma	(As above)	,
Insert single quotation marks	(As above)	‘γ and/or ’γ
Insert double quotation marks	(As above)	“γ and/or ”γ
Insert hyphen	(As above)	(hy)
Start new paragraph	⌐	⌐
No new paragraph	⌒	⌒
Transpose	⊔⊓	⊔⊓
Close up	linking ⁐ letters	⁐
Insert space between letters	⋏ between letters affected	#
Insert space between words	⋏ between words affected	#
Reduce space between letters	⊤ between letters affected	⊤
Reduce space between words	⊤ between words affected	⊤