Colour change: a novel biomarker indicating sublethal stress in the millipede *Julus scandinavius* (Diplopoda)

Michael Zanger and Heinz-R. Köhler

During investigations on the induction of the 70 kD stress protein family (hsp70, stress-70) in *Julus scandinavius* following exposure to different biohazards, the colour of the supernatant of the homogenate was closely correlated to the hsp70 level. Hsp70 has recently been shown to be a suitable biomarker for sublethal toxicity in soil animals. Control millipedes typically exhibited red or red-orange supernatants whilst the supernatant of starved or toxin-exposed diplopods was orange, orange-yellow, or even bright yellow. Based on these observations, a quantitative colour test was established which was found to be able to indicate the degree of stress situations caused by exposure to heavy metals (cadmium, zinc), organic pollutants (lindane, PCB 52), or by food deprivation in laboratory tests. It is suggested that this is caused by a breakdown of the red-orange bilirubins into orange-yellow urobilins.

Keywords: *biomarker, cuticle, Diplopoda, hsp70, pigments.*

Abbreviations: ci, colour index; csi, colour stress index; hsp, heat shock protein; PAGE, polyacrylamide gel electrophoresis; psi, protein stress index; SDS, sodium dodecyl sulphate.

Introduction

When exposed to contaminated food, most saprophagous soil invertebrates reduce their ingestion rates (e.g. Read and Martin 1990, Köhler *et al.* 1992a, Ullrich *et al.* 1993). Due not only to reduced ingestion rates, but also to diminished nutrient absorption efficiency the energy supply of diplopods is affected under such conditions (Köhler *et al.* 1992a). Subsequent to contaminant exposures, the activity and fecundity rates of diplopods are drastically reduced, which may when combined with similar adverse effects on the remaining soil biota cause a severe delay in organic matter breakdown and its subsequent mineralization in soils (Köhler *et al.* 1995). In terms of ecotoxicological monitoring, investigations on biochemical parameters indicating physiological stress in this widespread group of soil animals are promising to establish an early-warning biomarker-based test.

Therefore, in the present study we focused on the millipede *Julus scandinavius* (Diplopoda), which has been shown to be suitable to monitor sublethal environmental stress situations in different laboratory tests (Zanger *et al.* in press). An induction of stress proteins after exposure to harmful substances (e.g. heavy metals) has been previously shown for isopods and slugs (Köhler *et al.* 1992b, 1994), and for *J. scandinavius* (Zanger *et al.* 1994). In addition, other cellular and biochemical markers for toxicity assessment (ultrastructure, biotransformation enzymes) have been measured in *J. scandinavius*: preliminary results have revealed that this species reacts with an enhanced expression of CYP 1A-analogous protein to β-naphthoflavone treatment (Zanger, unpublished). Additionally, Köhler and Alberti (1992) and Berkus *et al.* (1994) have described cytopathological effects of heavy metal contamination in the midgut of diplopods.

Recently, whilst studying stress responses in *J. scandinavius*, we have noticed that the homogenate supernatants of animals exposed to noxious substances differed in colour from their controls. After centrifugation, the supernatants showed a red, red-orange, orange-yellow or even bright yellow colour. This observation led to the following questions:

(1) Which mechanism was responsible for this colour change, and was the effect correlated to the stress response or to another physiological parameter such as, for example, the extractable protein content of the organism?
(2) Is it possible to characterize the colour compound of the supernatants with respect to the current knowledge on arthropod cuticle pigments (Table 1)?
(3) Can we develop a simple and cost-efficient colour test to indicate environmental stress situations?

The objective of this work was to answer these questions.

METHODS

Conditions and contamination

Adults of millipede *Julus scandinavius* Latzel 1884 were collected from relatively uncontaminated forest sites (Hockenheim and Mauer, near Heidelberg, Germany: leaf litter metal concentrations at the Hockenheim site: <0.5 mg Cd per kg dry wt, 5–12.5 mg Pb per kg dry wt, <25 mg Zn per kg dry wt, Mauer site: <0.5 mg Cd per kg dry wt, 12.5–25 mg Pb per kg dry wt, 25–50 mg Zn per kg dry wt (Müller *et al.* 1987)).

For laboratory tests, diplopods were kept at 9°C in plastic boxes (11.5×11.5×4 cm^3), the bases of which were covered with moistened plaster of Paris. For laboratory contamination experiments, the animals (3–8 per box) were exposed to leaf litter material contaminated with either 51, 133, 216, 257, or 422 mg Cd^{2+} per kg dry wt (as $CdCl_2$), with 2518, 4705, or 22203 mg Zn^{2+} per kg dry wt (as $ZnCl_2$), or with 116 µg γ-hexachlorocyclohexane (lindane) per kg dry wt. For exposure to 0.1, 2.1 or 21 mg 2,2',5,5'tetrachlorobiphenyl (PCB 52) per kg dry wt, animals were kept on contaminated soil material. The substrate was always kept moist, controls were moistened with tap water. Whilst exposure time in the heavy metal experiments was 21 days, diplopods were exposed to PCB 52 and lindane for only 14 days. Additionally, samples were taken after 3 and 7 days of lindane exposure. Furthermore, some diplopods were kept under conditions of starvation for 21 days according to the time of exposure to heavy metals. At the end of the experiment all animals were frozen in liquid nitrogen.

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Pigment class	Pigment	Organism	Remarks on metabolism/colour	Source
Porphyrins	Porphyrins	Diplopods (cuticle)	Loosely bound to proteins and polysaccharides	Kennedy (1978)
	Coproporphyrin	*Polydesmus angustus* (cuticle)	Red	Needham (1968)
Ommochromes	Ommochromes	*Asellus* sp. (cuticle)		Needham and Brunet (1957)
	Xanthommatin	*Polydesmus angustus* (cuticle)	Yellow (oxidized form), red (reduced form)	Needham (1968), Kennedy (1978)
		Xanthommatin	Synthesized from tryptophane	Butenandt *et al.* (1954)
			Detoxication of high tryptophane levels	Linzen (1967)
Bilins	Bilinproteins	Insects (cuticle)		Urich (1990)
	Biliverdin	Mammals[a]	Green-yellow, breakdown product of porphyrins	Harper *et al.* (1986)
	Bilirubin	Mammals[a]	Red-orange, breakdown product of porphyrins	Harper *et al.* (1986)
	Uroblin	Mammals[a]	Orange-yellow, breakdown product of porphyrins	Harper *et al.* (1986)

Table 1. Selected information on presence, colour, and metabolism of pigments found in the cuticle of arthropods.

[a]Study used mammals for the characterization of different bilins.

Hsp70 analysis

After homogenization (potassium acetate 80 mM, magnesium acetate 4 mM, Hepes 20 mM, pH: 7.5) and centrifugation (20 000 *g*) of individual samples, the total protein concentration of the supernatant was determined according to Bradford (1976). Constant protein amounts (100 µg per lane) were separated by SDS–PAGE (12% acrylamide, 0.1% bisacrylamide (w/v), 2 h at 120 V), transferred to nitrocellulose (1 h at 100 mA) and the filters blocked for 2 h at 20° C in 50% horse serum in TBS (50 mM Tris pH 7.5, 150 mM NaCl). Subsequently, the nitrocellulose was incubated overnight at 20° C in primary antibody solution (mouse anti-human hsp70; dianova, Germany; dilution 1:5000 in 10% horse serum/TBS) and then washed in TBS. The secondary antibody (peroxidase-conjugated goat anti-mouse IgG; dianova, Germany; dilution 1:500 in 10% horse serum/TBS) was added for 2 h at 20° C. After washing the blot with TBS, the antibody complex was detected by 4-chloro(1)naphthole.

Grey value quantification of Western blot protein bands took place with the densitometric image analysis system Viper (Gesotec, Darmstadt, Germany) after background subtraction. Mean grey values of control specimens (n = 5–8) were set arbitrarily to 1.0, as a standard reference for the respective data obtained for the contaminated specimens.

Pigment change by different pH conditions

After homogenate centrifugation, 1 M HCl was added in excess to red, red-orange, or orange-yellow supernatants. The colour change was recorded optically and the reversibility of this process was investigated by the subsequent addition of 1 M NaOH. Vice versa, red, red-orange, or orange-yellow samples were subjected first to a pH increase (by 1 M NaOH) and subsequently to a strong decrease of pH (by 1 M HCl). The colour change was also recorded.

Photometry

After homogenization and centrifugation, 200 µl of either red-orange or yellow-green (after addition of NaOH, see above) supernatants were diluted 1:4 with acetate buffer solution (80 mM Mg-acetate, 5 mM K-acetate, 20 mM Hepes, pH: 7.5). The absorption of the diluted supernatants was measured photometrically in a spectrum between 200 nm and 700 nm (Ultraspec plus, LKB Biochrom).

Solubility of pigments

Red-orange homogenate supernatant (100 µl) was added to different solvents and the mixture was hand shaken for 1 min. Solubility of the pigment(s) in the solvents water, ethanol, benzoic acid methyl ester, benzene, xylol, and isopentan was recorded semi-quantitatively by visualization.

Colour index (ci) and relative colour stress index (rcsi)

Using a defined colour scale ranging from dark red (ci = 1) to bright yellow (ci = 7), the colour of the respective supernatants was estimated optically and classified by a colour index (ci, reference plates are shown in Figure 1). Colour was found to vary slightly according to the fresh weight of the animal. Therefore, a regression analysis of ci of control animals (n = 29) versus their fresh weight (mg) was conducted. Based on this analysis, the ci which can be expected for an ideal non-stressed specimen (ci_{exp}) with a fresh weight m (mg) can be calculated as:

$$ci_{exp} = 0.00754\, m + 1.91$$

For individual analysis of diplopods either exposed to a stressor or not, a colour stress index (csi) was calculated, which related the respective obtained colour (ci) of a tested specimen to the colour one should expect for controls of the same body size (ci_{exp}):

$$csi = ci \cdot ci_{exp}^{-1}$$

Since control specimens of a series of tested animals also varied slightly from the (theoretically) expected value (ci_{exp}), the csi was calculated not only for the contaminated specimens but also for every control group (csi_{ctrl}). The mean csi_{ctrl} of every control group was arbitrarily set to 1.0 as a reference for all directly related contaminated samples (csi_{test}). The quotient of both parameters equals the relative csi (rcsi):

$$rcsi = csi_{test} \cdot csi_{ctrl}^{-1}$$

Extractable protein content (P_{fw}) and relative protein stress index (rpsi)

Total protein concentrations of the supernatants were determined (Bradford 1976). According to the calculation of the relative colour stress index (rcsi), a relative protein stress index (rpsi) was defined. First, the extractable protein quantity per fresh weight of the animal P_{fw} (µg mg^{-1}) was calculated from the protein content of the supernatant P_{sp} (µg µl^{-1}), the volume of the extraction buffer solution V_{bs} (µl), and the fresh weight of the respective animal m (mg):

$$P_{fw}(\mu g\ mg^{-1}) = P_{sp}(\mu g\ \mu l^{-1})_{sp} \cdot V_{bs}(\mu l) \cdot m\ (mg)^{-1}$$

A linear regression analysis of extractable protein of controls (P_{fw}, n = 29) versus fresh weight (m) led to the calculation of the protein quantity (based on the fresh weight of the specimen) which one can expect to be extracted from control diplopods (P_{exp}):

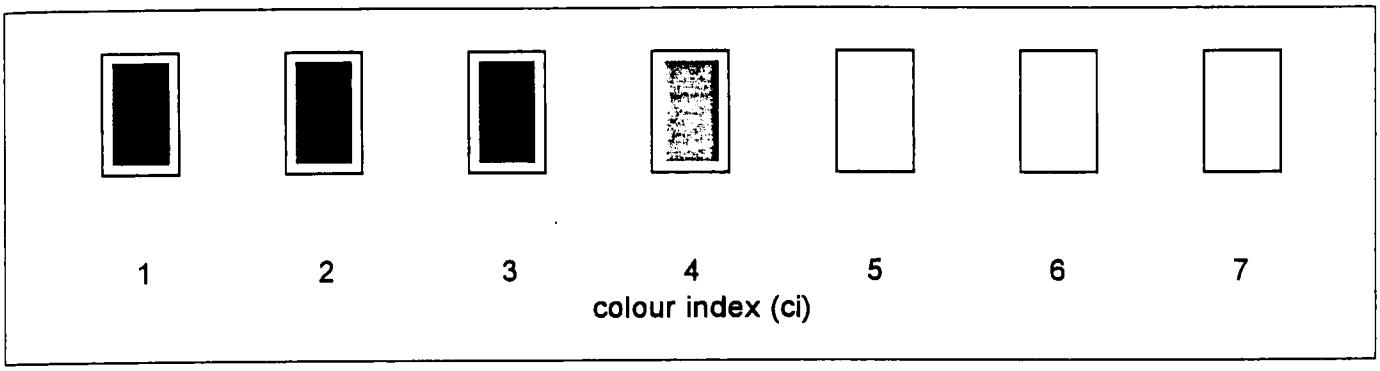

Figure 1. Reference colour scale and related colour index values (ci) used in the present study.

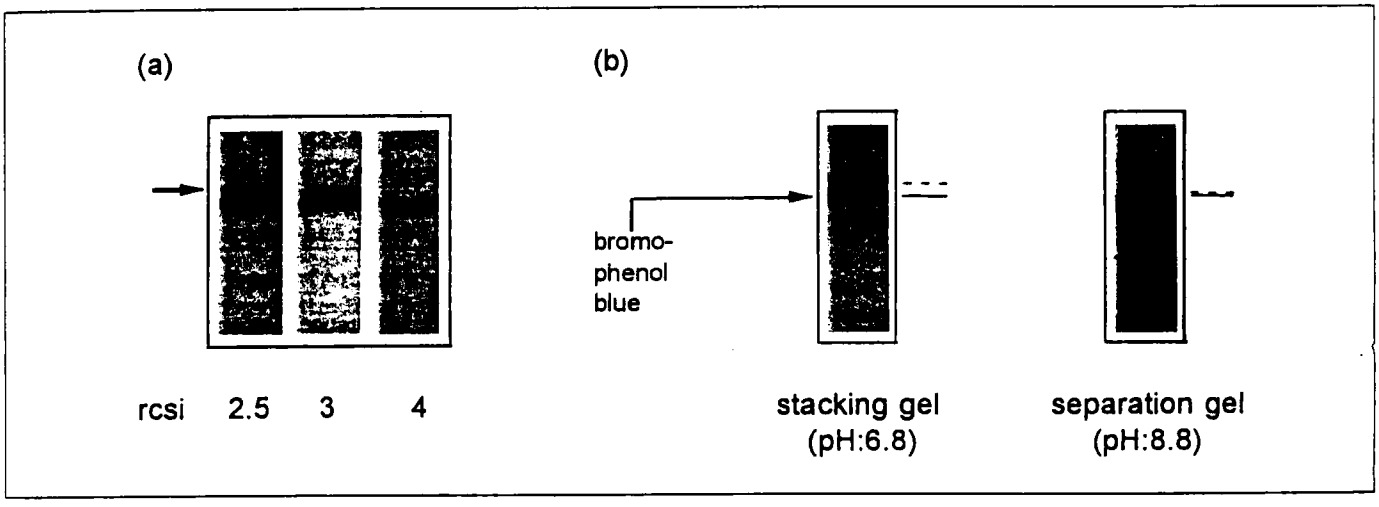

Figure 2. (a) Pigment band during electrophoresis. The arrow indicates the presence of a brownish band during SDS–PAGE. While in the case of red-orange supernatants (rcsi = 2.5) this coloured band was well-developed, only a faint band was found for orange supernatants (rcsi = 3.0), and was lacking for orange-yellow or bright yellow ones (rcsi = 4.0 or higher). (b) During electrophoresis, in the stacking gel, the brownish band (interrupted line) is running slightly above the marker band of bromophenol blue (solid line). When entering the separation gel, it increased running speed and approached the marker band (692 Da).

$$P_{exp} = 0.155\ m + 39.234$$

Subsequently, we calculated a protein stress index (psi), relating the extracted protein of test animals (P_{tw}) to P_{exp} for control animals of the same body weight:

$$psi = P_{tw}(\mu g\ mg^{-1}) \cdot P_{exp}(\mu g\ mg^{-1})^{-1}$$

In order to consider and make allowance for slight variations within a series of control specimens, the psi was always calculated for every series and its control. The mean psi of each control group (psi_{ctrl}) was arbitrarily set to 1.0 as a reference for all other data (psi_{test}) of the same test series. The relative protein stress index (rpsi) was calculated by:

$$rpsi = psi_{test} \cdot psi_{cont}^{-1}$$

Statistics

The observations obtained in the present study resulted in the assumption that *J. scandinavius* reacted to toxic stress in a threshold type of response: either increased hsp70 production is induced by the stressor or there is no response. Thus, statistically, a normal Gauss distribution of the measured grey values cannot be implied. Therefore, the Mann–Whitney–Wilcoxon test which refers also to non-Gauss distribution of values was applied to show significant difference between controls and the related contaminated animals: $p > 0.05$ (not significant, $^{-}$), $0.05 > p > 0.025$ (slightly significant, *), $0.025 > p > 0.01$ (significant, **), $0.01 > p > 0.001$ (highly significant, ***).

Results

Hsp70 induction and presence of pigments

Exposure of the millipede to the elevated heavy metal concentrations induced elevated levels of hsp70 proteins. Whilst exposure to increasing zinc concentrations resulted in a steadily increasing hsp70 level, exposure of diplopods to cadmium showed an optimum curve for hsp70 induction, which reached a maximum for animals exposed to leaf litter containing 133 mg Cd^{2+} per kg (see Figure 5). A single surviving animal responded to extreme feeding conditions (422 mg Cd^{2+} per kg) with a comparatively high hsp70 level (see Table 2).

Stress condition	n	rgv	rcsi	rpsi
Control for Cd/Zn	7	1.00±0.39	1.00±0.26	1.00±0.19
51 mg Cd	5	1.71±1.26	0.98±0.13	0.95±0.33
133 mg Cd	3	3.27±3.80	1.19±0.57	0.50±0.38*
216 mg Cd	2	1.56[1]/3.38[1]	0.95[1]/1.46[1]	0.75[1]/0.49[1]
257 mg Cd	4	2.30±1.37	1.13±0.31	0.59±0.24**
413 mg Cd	1	11.77[1]	2.24[1]	0,74[1]
2518 mg Zn	4	1.49±0.94	0.86±0.03	0.77±0.22
4705 mg Zn	3	1.63±1.33	1.03±0.32	1.17±0.17
22203 mg Zn	3	8.18±5.95*	2.09±0.96*	0.91±0.28
Control for PCB 52	8	1.00±0.00	1.00±0.22	1.00±0.25
0.1 mg PCB52	7	1.00±0.00	1.08±0.19	0.98±0.19
2.1 mg PCB52	7	1.00±0.00	1.00±0.12	0.81±0.18
21 mg PCB52	7	1.00±0.00	1.21±0.14*	0.74±0.19
Control for γHCH	6	1.00±0.00	1.00±0.42	1.00±0.52
116 µg γHCH (3d)	5	1.00±0.00	1.29±0.4	1.19±0.33
116 µg γHCH (7d)	5	1.00±0.00	1.10±0.56	1.07±0.69
116 µg γHCH (14d)	7	1.00±0.00	1.28±0.44	1.11±0.25
Control for starvation	8	1.00±0.00	1.00±0.31	1.00±0.14
Artificial starvation	9	1.00±0.00	1.29±0.41	1.09±0.24

Table 2. Condensed overview upon the correlation of hsp70 induction, colour and extractable protein content. Means±standard deviations. Toxin concentrations related to (kg dry wt)[-1].

Key: n number of test animals; γHCH, lindane; rgv, relative grey value of the hsp70 band; rcsi, relative colour stress index; rpsi, relative protein stress index.

[1]Single data.
Significance levels: $*0.05 > p > 0.025$, $**0.025 > p > 0.01$ (Mann–Whitney–Wilcoxon test).

When relating the hsp70 levels to the colour of supernatants, we found that non-stressed animals always showed a red or red-orange colour. This corresponded to the appearance of a reddish to brownish band during SDS–PAGE; this band ran immediately behind the marker band of bromophenol blue (3′-3″-5′-5″-tetrabromophenol sulphonphthalein). Stressed diplopods showed an orange-yellow or bright-yellow colour of the supernatants and at most a very faint brownish band was visible during SDS–PAGE (Figure 2(a)).

Individuals of *J. scandinavius* exposed to PCB 52 or lindane failed to induce a hsp70 response. Nevertheless, the colour of the supernatants was in most cases red or red-orange, but orange-yellow samples also occurred. This again was in accordance with the presence of the brownish band during electrophoresis (Figure 2(a)).

Characterization of pigments

Colour and absorption

When investigating the absorption spectra of red-orange supernatants obtained directly from homogenates, peaks were demonstrated at 224 nm and at 289 nm. The green-yellow pigment which had been altered by NaOH showed only one peak at 227 nm. Generally, in both cases, absorption between 350 nm and 500 nm was weak and dropped to nearly zero for wavelengths between 500 nm and 700 nm (Figure 3).

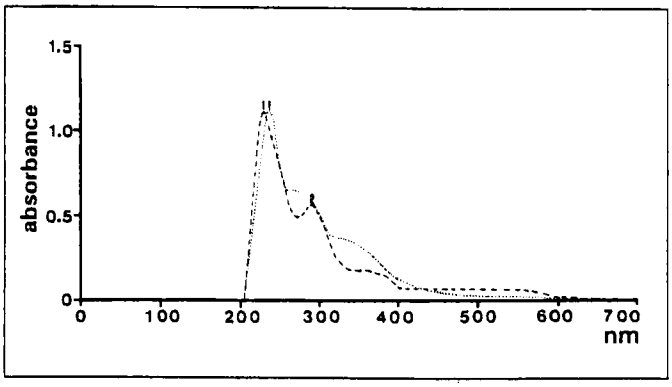

Figure 3. Absorption spectra of red-orange supernatant (dashed line) and of the green-yellow pigment (dotted line) altered by NaOH. Absorption peaks were only found between 200 and 300 nm. For wavelengths > 400 nm, absorption dropped to nearly zero.

Influence of the pH

The alteration of red or red-orange pigments by the addition of 1 M HCl was visible by a change to an orange-yellow colour which did not change with further additions of 1 M HCl. Subsequent addition of 1 M NaOH resulted in a colour change to green-yellow. This effect was found to be reversible by addition of more 1 M HCl (see Figure 4).

Heat resistance

When preparing samples for SDS–gelelectrophoresis, the supernatants were heated at 95° C for 5 min. After re-cooling them, the pigments of the supernatants remained stable.

Molecular weight

During SDS–PAGE, in the stacking gel (pH: 6.8), a reddish to brownish band running slightly behind the marker bromophenol blue (3′-3″-5′-5″-tetrabromophenol sulphonphthalein, 692 Da, Figure 2(b)) appeared in the samples without clearly increased hsp70 protein levels (see above). This pigment approached the marker after entering the separation gel (pH: 8.8) and, subsequently, remained very slightly behind the bromophenol blue band (Figure 2(b)). This change in speed led to the suggestion that acid groups of the pigments may possibly have been deprotonated in the separation gel resulting in a stronger negative charge of the molecules and an increased speed. The molecular weight of the pigments was estimated to be about 700 Da.

Solubility

The pigment of the red-orange homogenates was found to be insoluble in neutral organic substances such as isopentane, xylol or benzene. On the other hand, it was soluble in a weak organic acid (ethanol) and even in water (Table 3).

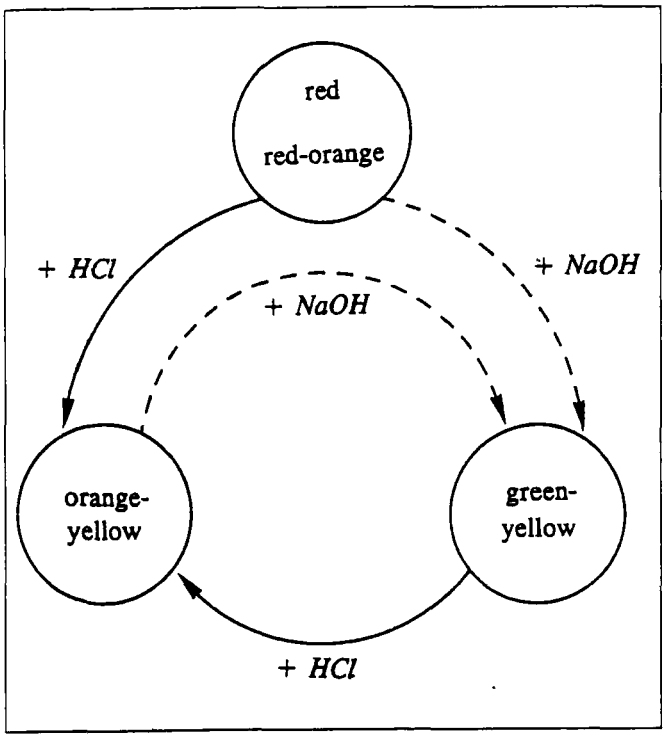

Figure 4. Schematic representation of the colour change of homogenate supernatant according to different pH conditions. Solid lines indicate the addition of HCl, dashed lines the addition of NaOH.

Colour change induced by different stressors

Non-stressed animals showed red, red-orange, or sometimes orange, but never orange-yellow or bright-yellow supernatants. The colour correlated with the weight (age) of diplopods: smaller (younger) animals as a rule gave a red colour, larger (older) specimens red-orange or an orange colour. Based on these observations the ci_{exp} and, subsequently, rcsi were calculated for all samples (Table 2, Figure 5).

After exposure to increasing cadmium concentrations a gradual change in colour compared with control conditions appeared. Only low Cd^{2+} contamination (51 mg kg^{-1}) led to red-orange supernatants whilst higher Cd^{2+} concentrations resulted in a change to an orange or even yellow colour.

When exposed to zinc, diplopods showed a similar colour pattern. With exposure to the lowest applied concentrations all individuals had red supernatants, increasing zinc concentration resulted in red-orange or bright yellow supernatants.

The response to PCB 52 was not as prominent compared with the metal effects. Low PCB 52 concentrations did not result in a significant change of the supernatant colour. Only following exposure to the highest applied PCB 52 concentration was a clear colour change to orange supernatants observed.

In contrast to all other exposure experiments, the exposure time–response relationship was investigated for lindane. After an exposure time of just 3 days, a colour change towards orange supernatants was found in the animals, which did not change significantly during the following exposure time.

Diplopods kept under artificial starvation conditions for 3 weeks also showed a fading of red pigments in their supernatants which, however, was not as strong as in the highly metal-exposed animals.

Solvent	Solubility
Water	+
Ethanol	+
Benzoic acid methyl ester	(+)
Xylol	-
Benzene	-
Isopentane	-

Table 3. *Semi-quantitative estimation of the solubility of the red-orange pigment in different solvents.*

Key: +, soluble; (+), poorly soluble; -, insoluble.

Protein content

While animals exposed to cadmium or PCB 52 showed a decrease in the amount of extractable protein, this effect did not appear significant for animals kept under starvation conditions, for animals contaminated with zinc, and for lindane-exposed diplopods (Table 2).

Interrelationship of hsp70, colour, and protein contents

Based on the present observations, the following parameters coincided with one another:

- Non-stressed diplopods (e.g. controls) which showed at most very weak bands of hsp70 (or no band at all) in the Western blot presented red or red-orange supernatants.
- Contamination with heavy metals inducing hsp70 expression resulted in a change of supernatant colour from red or red-orange to orange and finally to bright yellow. An increase of the relative csi up to 200% or more compared with controls was calculated for some of these specimens.
- Whilst exposure to organic contamination (PCB 52, lindane) led to an elevated relative csi value, an induction of hsp 70 could not be detected. This possibly could be explained with the induction of biotransformation enzymes which may protect the intracellular milieu from protein degradation (see also 'Discussion').
- Artificial starvation also caused colour modifications of the supernatant despite an accompanying lack of hsp70 induction. The relative csi, however, did not reach the level shown by animals exposed to the higher heavy metal concentrations.
- The reddish to brownish band which occurred in the gels during electrophoresis was more prominent in samples showing low or no hsp70 expression. It was assigned to be the pigment which stained homogenates of non-stressed animals red or red-orange.
- Exposure to cadmium resulted in a clear decrease in the protein contents of the animals.

Discussion

As previously shown for aquatic organisms (summarized by Sanders 1993), the suitability of the hsp70 response to monitor

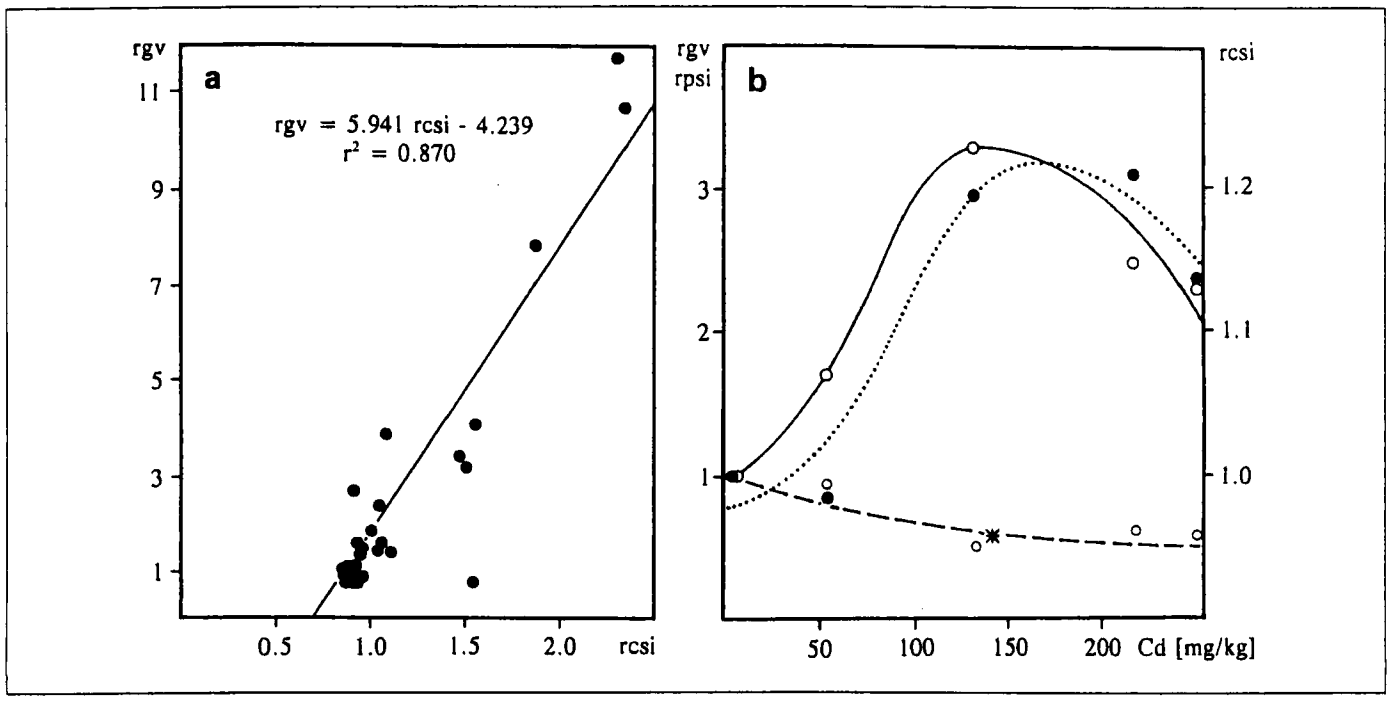

Figure 5. (a) Correlation of the stress-70 protein response and the colour change (rcsi values) of supernatants of cadmium- or zinc-treated animals and their respective controls (rgv: relative grey value of the stress-70 protein bands). Linear regression analysis by TableCurve (Jandel Scientific). (b) Concentration–response of the hsp70 protein expression (rgv, big circles, solid line, left scale), relative colour stress index (rcsi, black dots, dotted line, right scale) and relative protein stress index (rpsi, small circles, interrupted line, left scale) of *J. scandinavius* exposed to cadmium (means and regression curves). Significance levels: $^{*}0.05 > p > 0.025$ (Mann–Whitney–Wilcoxon test).

stress situations caused by environmental hazards in soil invertebrates has recently been demonstrated. When either natural populations or transgenic strains of soil animals have been investigated, however, they usually only express elevated hsp70 levels when exposed to harmful substances (Köhler *et al.* 1992b, Eckwert *et al.* 1994, Guven *et al.* 1994, Zanger *et al.* 1994). Based on the fact that the hsp70 level indicates the degree of proteotoxic (protein-decaying) impact on a specimen (Parsell and Sauer 1989, Perisic *et al.* 1989, Sorger and Nelson 1989, Craig and Gross 1991), the present study has led to three main conclusions regarding the possibilities of indicating contaminated locations with the biomarker 'colour change' in *Julus scandinavius*:

(1) A correlation between hsp70 expression and the colour of the supernatants was apparent. Non-stressed diplopods showed (in relation to their individual weight) a red or red-orange colour and no or only a slightly increased hsp70 level detectable by Western blotting. In contrast, diplopods expressing higher hsp70 levels exhibited orange, orange-yellow, or bright yellow colours in their centrifuged homogenates. Comparison of the relative csi values with the relative grey values of their corresponding hsp70 bands yielded a strong correlation between these two parameters (Figure 5(a)). Additionally, the reaction pattern of both parameters to increasing heavy metal stress was found to be very similar. The expression of hsp70 as well as of the relative csi followed an optimum curve in relation to increasing cadmium exposure. Whilst lower cadmium concentrations led to an increase of relative grey values for hsp70 and relative csi, both parameters decreased when animals were exposed to very high cadmium concentrations. This reduction in hsp70 expression is most likely a consequence of severe pathological damage which high concentrations of heavy metals have been able to cause in the diplopod's midgut and the associated hepatic cells (Köhler and Alberti 1992, Berkus *et al.* 1994). Typical cytopathological symptoms are related to osmotic changes in the resorptive epithelium and to alterations of the cytoskeleton which lead to dislocation of cellular organelles (Köhler and Alberti 1992). Contrary to the results obtained for cadmium exposure, both hsp70 induction and relative csi in animals fed zinc-contaminated leaf litter, followed a *power* function. Only an exposure to 22203 mg Zn^{2+} per kg dry weight resulted in a massive hsp70 expression and correlated with a clear colour change of the supernatant. Since zinc forms an essential part of carbonic anhydrase which is prominent in animals with calcified cuticles (Maren 1967), diplopods seem able to tolerate moderate zinc contamination. If cellular mechanisms of detoxification are overcharged, however, the presence of essential metals like zinc can cause toxic effects (Hopkins 1989).

Exposure of *J. scandinavius* to organic compounds (lindane, PCB 52) did not lead to elevated hsp70 levels but did result in increase relative csi. Apparently, proteotoxicity following lindane or PCB 52 seems to be comparatively low which suggests the presence (and induction) of biotransformation enzymes in diplopods. Recently the presence and the activity of compounds of the cytochrome P450 system (CYP 1A-like gene products, and ECOD activity) in *J. scandinavius*

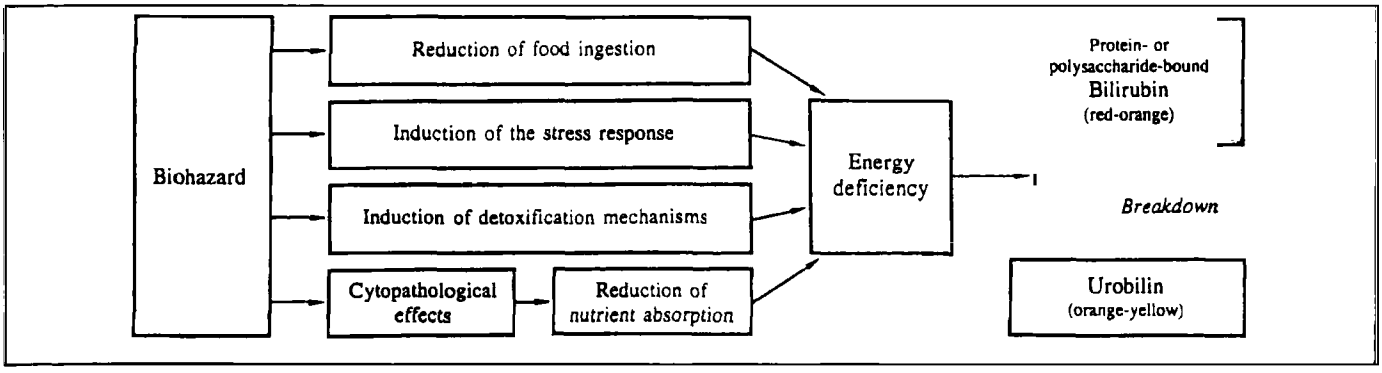

Figure 6. Proposed model explaining the observed effects. In reaction to different biohazards diplopods usually show different physiological alterations which – in total – lead to a deficiency in energy supply. Subsequently, this lack of energy forwards the breakdown of protein- or polysaccharide-bound bilirubins into urobilin in or close to the cuticle.

following β-naphthoflavone contamination has been measured (Zanger, unpublished). Equipped with these enzymes diplopods may detoxify low concentrations of organic substances before they induce intracellular protein degradation and the subsequent hsp70 response. This seems to be also true for food deprivation which leads to a mobilization of reserve substances but apparently does not result in an intracellular accumulation of degraded protein. Since the induction of detoxification processes as well as the survival of starving situations requires the mobilization of energy any change of rcsi most probably indicates a change in the diplopod's energy status. Thus, the relative csi is an easily detectable and convenient supplement to toxicity studies based on hsp70 which itself exclusively indicates proteotoxic situations.

(2) The stress-induced colour modifications are likely to be due to the mobilization of reserve substances from the cuticle of Julus scandinavius. For diplodods, Zhulidov and Dubova (1988) and Köhler *et al.* (1992a) found reduced assimilation rates in *J. scandinavius* and other diplopod species fed metal-contaminated food. To overcome this energy deficiency, a mobilization of reserve substances should be considered; this has been shown, for example, by Berkus (1994) who found a reduction in incellular glycogen stores when *J. scandinavius* was fed with heavy metal-contaminated leaf litter. Seifert and Rosenberg (1977) have also described the same effect for diplopods kept under starvation conditions. In order to identify the colour substance in the homogenates, we focused on porphyrin and its derivatives. Different authors have described porphyrins as being widely distributed pigments in the cuticle of diplopods (Needham 1968, Kennedy 1978). Since Kennedy (1978) suggested that porphyrins and their derivatives are loosely bound to proteins or polysaccharide in the cuticle, and Urich (1990) also described bilin-bound proteins as being typical pigments of insects, we considered that a breakdown of bilin-associated nutrients would be able to compensate for the energy deficiency induced by adverse feeding conditions. The reduction of extractable protein contents of animals contaminated with cadmium and PCB 52 was interpreted as additional evidence for this explanation.

To summarize, energy deficiency primarily causes a mobilization of cellular glycogen (or even lipid) stores, which were not involved in the composition of cuticle pigments. After exhausting these stores, the maintenance of adverse conditions (starvation, food contamination, etc.) most likely leads to a mobilization of polysaccharide and protein-associated compounds from the cuticle. The results from the values of extractable protein lead us to assume an earlier breakdown of polysaccharide-pigment compounds than of protein-bound substances.

(3) Using chemical and physical methods, the colour compounds of supernatants could be identified as porphyrin derivatives. Characteristic references to this conclusion are (a) the estimated molecular weight of the pigments of approximately 700 Da, (b) acid behaviour during electrophoresis, (c) the insolubility in neutral organic solvents, (d) the heat resistance against temperature of up to 95°C, (e) the pH-dependent colour pattern, and (f) the measured extinction peaks between 200 and 300 nm.

The absence of a soret peak at about 400 nm, which is typical for circular closed porphyrins, indicates that the pigments are longitudinally stretched tetrapyrroles (bilins) (Falk 1975). The rupture of the porphyrin macrocycle generally leads to a reduction in absorption in the visible spectrum and thus the main absorption peaks only remain in the UV-spectrum (Fischer and Stern 1940).

The extracted pigment is unlikely to be xanthommatin (ommochrome) as it becomes red when reduced (homogenates of diplopods were typically orange-yellow) and xanthommatin is unstable at high pH (Linzen 1967).

Linking the results of the present study to observations of other authors we developed a simple hypothetical model of the dependencies of physiological changes and different colours in the diplopod *J. scandinavius* (Figure 6).

Based on the present results, we propose that control diplopods mainly contain red-orange bilirubin, which breaks down into orange-yellow urobilin in stressed (energy-deficient) animals. However, it remains unclear whether these bilins are present in their reduced form *in vivo* or gain electrons during the homogenization process which would then indicate a divergent redox-level of different homogenates.

In this study we presented a novel biomarker; '*the colour of supernatants*', which is very practical to measure and sufficient for indicating environmental stress. Although the

colour change is also induced by starvation to a minor degree, preliminary field studies in metal-contaminated areas showed the capacity of this effect to indicate adverse situations under field conditions (Zanger and Köhler, unpublished). Thus, presumably, heavy metal exposure and insufficient nutrition of the diplopods may coincide in most cases. To consider individual variation in the nutritional status of the animals, however, a statistically sufficient number of replicates is required for routine field determination.

Closer elucidation of the physiological processes involved in the colour change phenomenon and the development of a 'test-kit' for field studies are future aims.

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