Dietary acquisition of photoprotective compounds (mycosporine-like amino acids, carotenoids) and acclimation to ultraviolet radiation in a freshwater copepod

Robert E. Moeller, Shawna Gilroy, Craig E. Williamson, and Gabriella Grad
Department of Earth and Environmental Sciences, Lehigh University, 31 Williams Drive, Bethlehem, Pennsylvania 18015

Ruben Sommaruga
Laboratory of Aquatic Photobiology and Plankton Ecology, Institute of Zoology and Limnology, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria

Abstract

We experimentally tested the hypothesis that accumulations of dietary compounds such as carotenoids or UV-absorbing mycosporine-like amino acids (MAAs) protect against natural levels of ultraviolet radiation (UVR). A calanoid copepod, *Leptodiaptomus minutus*, was collected from a relatively UV-transparent lake in Pennsylvania where levels of copepod MAAs and carotenoids vary during the year (MAAs high/carotenoids low in summer). Animals raised in the laboratory under different diet/UVR treatments accumulated MAAs from an MAA-producing dinoflagellate but not from a cryptomonad that lacks them. The acquisition efficiency increased under exposure to UVR-supplemented photosynthetically active radiation (PAR, 400–700 nm), yielding MAA concentrations up to 0.7% dry weight compared with only 0.3% under unsupported PAR. Proportions of individual MAAs differed between the animals and their diet. Shorter wavelength absorbing palythine and shinorine (*λ* max 320 and 334 nm, respectively) were disproportionately accumulated over usujirene and palythene (*λ* max 359 nm). Carotenoids accumulated under UVR exposure (to 1% dry weight) when dietary MAAs were not available. Tolerance of ultraviolet-B (UV-B) radiation was assessed as LE50 s (UV exposure giving 50% mortality after 5 d) following 12-h acute exposure to artificial UV-B radiation. LE50 s increased 2.5-fold for UV-acclimated, MAA-rich animals, but only 1.5-fold for UV-acclimated, carotenoid-rich animals. Compared with carotenoids, MAAs offer this copepod a more effective photoprotection strategy, potentially as important as photorepair of DNA damage, to promote tolerance of natural levels of UV-B radiation.

Zooplankton living in surface waters potentially encounter harmful levels of ultraviolet radiation (UVR), especially UV-B (Williamson et al. 1994). Assessing the effect on natural populations, however, requires complex integration of spectral sensitivity, incident radiation, and active or passive movements within the depth-gradient of UVR intensity (Browman et al. 2000). UVR often is rapidly attenuated with depth in freshwaters (Morris et al. 1995). Many organisms physically avoid harmful intensities, including migrating copepods (Alonso et al. 2004) and other zooplankton. Moreover, zooplankton and other organisms possess biochemical defenses against UVR; these variously intercept UV photons, neutralize oxidizing photoproducts, or repair damage to DNA and other cell constituents (Mitchell and Karentz 1993; Banaszak 2003; Hessen 2003). Some zooplankton are more tolerant of UVR than others, for example, copepods compared with cladocerans (Leech and Williamson 2000; Goncalves et al. 2002), or, among *Daphnia*, melanic compared with nonmelanic (Hessen 2003) and epilimnetic compared with metalimnetic populations (Siebeck and Böhm 1994). These differences raise a key issue in understanding an organism’s distribution within the water column or among lakes of contrasting UV transparency. Are observed tolerances a determinate factor for distributions or merely aclimatory responses that reflect them?

The expression of various photoprotective compounds is a recognized response to high irradiance, and likely is subject to aclimatory regulation. Carotenoids are familiar as orange (blue-light absorbing) pigments that protect against high light intensities in many organisms (Goodwin 1986), including copepods (Hairston 1976). They often function as antioxidants as well as light-blocking pigments (Edge et al. 1997), which accounts for their apparent effectiveness against UV wavelengths (Ringelberg et al. 1984). Another class of photoprotective compounds, the UV-absorbing mycosporine-like amino acids (MAAs), directly screens out UVR in many algae and aquatic invertebrates (reviewed in Karentz 2001; Shick and Dunlap 2002), including freshwater copepods (Sommaruga and García-Pichel 1999). As with carotenoids, zooplankton presumably must acquire MAAs from their diet (Newman et al. 2000; Hellbling et al. 2002). Invertebrates and other animals lack the shikimate synthetic pathway required for de novo MAA synthesis (Karentz 2001; Shick and Dunlap 2002). Dietary scarcity of these compounds within planktonic food webs could potentially constrain UVR defenses dependent on them.
Here we address several key issues concerning the presumed photoprotective role of MAAs in a freshwater calanoid copepod, *Leptodiaptomus minutus*. These include (1) dietary dependence, (2) accumulation as an acclimatory response to UVR stress, (3) retention during dietary unavailability, (4) complementariness to other defenses—notably carotenoid accumulation and DNA photorepair—and (5) effectiveness as a UV-B defense. *L. minutus* was selected for this study because we wish to understand how it is able to tolerate ambient UVR better than the cladoceran *Daphnia catawba* in a Pennsylvania lake (Williamson et al. 1994).

The copepod is most tolerant of UV-B in summer (Stutzman 1996, 2001; Carroll and Shick 1996; Adams et al. 2001) showed that MAAs acquired from macroalgae could enhance tolerance to UVR in controlled laboratory experiments. Marine krill likewise take up MAAs from phytoplankton (Newman et al. 2000). Here, our strategy was to raise copepods in the laboratory under different diet and UVR conditions, then compare resultant content of MAAs and carotenoids to UV-B tolerance in acute toxicity bioassays. Finally, animals differentially acclimated to UVR in the laboratory were exposed over several days in the surface water of their native lake to confirm the relevance of laboratory results to natural light conditions.

**Methods**

The experiments described here involve raising copepods under different diet and irradiance conditions then evaluating their UV tolerance. Common features of the experiments are described first (source of animals, diet and irradiance conditions, protocol for testing UV tolerance, analysis of photoprotective compounds). The design and specific details of the several experiments follow.

**Source of copepods**—The copepod *L. minutus* was collected from Lake Giles (Pike County, Pennsylvania; 41°22′N, 75°05′W), where it is a major component year-round of the macrozooplankton. Animals from daytime vertical net hauls (11 dates, September 2001–November 2002) were collected for analyses of photoprotective compounds. During thermal stratification (May–October) sampling was restricted to the epilimnion (upper 5–10 m of the 24-m deep lake) to obtain animals potentially exposed to UVR. Laboratory cultures initiated from the October 2001 collection were maintained for 10 months and used for all but one experiment, which used animals raised from adults collected in November 2002. Lake Giles is a natural oligotrophic, soft-water lake (pH 5.4–5.7) with relatively high UV transmissivity in midsummer (Morris and Hargreaves 1997).

**Laboratory culture of copepods**—Animals were raised and maintained at room temperature (20–24°C) under fluorescent lights (14:10 h light:dark cycle) on a laboratory light-table (see next section). They were fed a mixture of two cultured algae: the cryptomonad *Cryptomonas reflexa* Skuja—or *Campylomonas reflexa* (Sskuja) Hill—isolated from a Pennsylvania pond (Williamson and Butler 1987) and the dinoflagellate *Peridinium inconspicuum* Lemm. from the University of Texas culture collection (UTEX LB 2255). The dinoflagellates produces MAAs when grown under fluorescent cool-white light, with or without added UVR. We have not attempted to quantify differences in MA production under different irradiances. The *Peridinium* supports good copepod growth but poor reproduction unless supplemented with *Cryptomonas*, which does not produce MAAs, even when grown under UVR. For these experiments, *Peridinium* was grown under UV-supplemented light and *Cryptomonas* under cool-white fluorescent light only. The proportions of the algal foods and the intensity of UVR during copepod growth varied among the experiments reported here. Algae were added daily. The amount was continuously adjusted for the algal foods and the intensity of UVR during copepod growth varied among the experiments reported here. Algae were added daily. The amount was continuously adjusted for the next feeding and (b) were sufficient to support rapid copepod growth and significant adult reproduction (clutches of 4–8). Animals were maintained in borosilicate culture dishes with borosilicate lids (Pyrex Petri dish bottoms) in 0.2-μm filtered Lake Giles water and changed at 4–7 d intervals. Contaminants included bacteria, algae, and protozoa introduced with the copepods, but the added algae were unquestionably the predominant food.

**Light conditions during growth**—Irradiance was provided by four 40-W cool-white fluorescent tubes combined with two 40-W UVA-340 fluorescent tubes (Q-Panel). The UVA lamps were positioned directly above the cultures, the cool-white lamps angled above and flanking the cultures. Low, medium, and high UVR intensities were obtained by varying dish-to-lamp distance (Table 1). Cultures intended for the higher intensities were moved progressively closer to

| Table 1. Irradiance in laboratory experiments compared to solar irradiance. Copepods were raised in ultraviolet radiation (UVR) plus photosynthetically active radiation (PAR) (UVR growth treatment) or PAR only (PAR) at different distances from the fluorescent ultraviolet A (UVA-340) and cool-white lamps. |
|--------------------------|-------------------------|-------------------------|--------------------------|
|                         | UV-B                    | UV-A                    | PAR                      |
|                         | (W m⁻²)                 | (W m⁻²)                 | (µmol quanta m⁻² s⁻¹)    |
| Solar (July midday)     | 2.21                    | 40.0                    | 1800                     |
| Growth treatment        |                         |                         |                          |
| UVR                     |                         |                         |                          |
| 10 cm                   | 0.806                   | 8.43                    | 90                       |
| 17 cm                   | 0.571                   | 5.98                    | 107                      |
| 34 cm                   | 0.315                   | 3.29                    | 93                       |
| PAR                     |                         |                         |                          |
| 10 cm                   | 0.0003                  | 0.083                   | 87                       |
| Phototron*              |                         |                         |                          |
| – PR                    | 1.84                    | 1.99                    | <0.1                     |
| + PR                    | 1.99                    | 3.91                    | 6                        |

* Three ultraviolet (UVB 312) lamps (aged acetate) ± photorepair radiation (PR), without neutral density filters.
the lamps in 2–3 equal steps (distance) of 4–6 d duration. Intensity and spectral composition of irradiance were measured at 1-nm resolution with a UV–photosynthetically active radiation (PAR) spectroradiometer (Williamson et al. 2001). Growth irradiance is compared with natural sunlight in Fig. 1 (spectra smoothed by 3-nm running average) and Table 1 (integrated for UV-B, UV-A, and PAR wavebands). Growth irradiance was adjusted for absorption by the boro-silicate dishes ($A_{304nm} = 304$ nm, PAR transmission > 90%). Laboratory PAR treatments also were filtered through UV-absorbing acrylic (Acrylite OP-2, Cyro Industries; $A_{400nm} = 400$ nm, PAR transmission > 90%). Solar irradiance is the incident 4-h midday average for sunny July days near Lake Giles. The spectrum was generated to fit measured irradiances at 305, 320, 340, and 380 nm (Biospherical Instruments GUV 500) using the program RTBasic (C.R. Booth, Biospherical Instruments).

Testing UV tolerance—Copepod survival was monitored following 12-h exposure to UVR in a “UV lamp phototron” (Williamson et al. 2001). This incubation system exposes rotating quartz dishes (8–12 animals in 30 ml of medium) to UV-B plus UV-A from above and to UV-A plus PAR (and some additional UV-B) from below. UV-B was provided by 3 UV-312 nm lamps (Spectroline XX15B; Spectronics). The UV-C absorbing plate built into the lamp housing was supplemented with fresh cellulose acetate film for each 12-h exposure. The resultant UVR output, measured with our spectroradiometer (Fig. 1, Table 1), lies half in the UV-B range (280–320 nm) and half in the UV-A range (320–400 nm). Exposure levels were manipulated by placing neutral density filters (metal screens) of known proportional transmission ($f$) on top of the dishes. UVR exposure (or dose) is defined by UVR irradiance ($I_{UV}$), the filter factor ($f$), and duration of exposure (12 h): exposure (kJ m$^{-2}$) = $f \times I_{UV}$ (W m$^{-2}$) × 0.001 (kJ s$^{-1}$ W$^{-1}$) × 12 (h) × 3,600 (s h$^{-1}$). Note that all references to UVR exposures in the phototron experiments refer to total UVR from the Spectroline lamps, ignoring additional, predominantly longer wavelength UVR from the lamps below. The irradiance from below (80 W each of cool-white and UVA-340 lamps) provides PAR and UV-A, with a small amount of additional UV-B. This irradiance potentially enables photorepair, or photoreactivation: the light-dependent (370–450 nm) enzymatic repair of certain types of DNA lesions (Mitchell and Karentz 1993; Banaszak 2003). In two phototron experiments (phototrons 2, 5) opaque discs were inserted below a subset of the dishes, giving a contrast of treatments with and without photorepair ($\pm$ PR). Since some UV-A > 370 nm is produced by the UV-B lamps themselves (Fig. 1), the significance of photorepair may have been somewhat underestimated by this technique.

Following exposure, animals were incubated in the dark. They were fed *Cryptomonas* and examined daily for 5–7 d. A parallel set of control dishes was maintained in the dark throughout the experiment. All five phototron experiments were carried out at 20 ± 1°C.

The phototron experiments are examples of acute toxicity bioassays (Rand and Petrocelli 1985). The objective is to establish the 12-h cumulative UVR exposure (or dose) that causes 50% cumulative mortality over some fixed time interval, in this case 5 days. This median lethal exposure (5-d $LE_{50}$) was computed from linear regression of logit (survival) on log$_{10}$ (exposure). Taking $q$ as proportion surviving in a particular dish and rescaling the logit transformation for convenience, logit’ (survival) = 0.5 logit ($q$) + 5 = 0.5 ln [g/(1 − g)] + 5.

Typically, each exposure treatment consisted of five replicate dishes of ca. 10 animals each. Raw mortality in each dish was adjusted for “natural mortality” (Abbott’s formula) in the treatment showing lowest mortality—in principle the control treatment, but in practice sometimes the lowest UV treatment. Natural mortality at 5 days was always ≤ 10%. Logit values are undefined when survival is 0 or 100%. Thus data points for dishes with full mortality or full survival were adjusted slightly by adding or subtracting 0.5 animal, respectively. No more than two such adjusted values were used in statistical analyses. The 95% confidence interval for $LE_{50}$ was estimated graphically from linear $y$-on-$x$ regression (Sigma-Plot ver. 5.0, SPSS) as the $x$-axis projection of the 95% prediction envelope at the $y$ value of interest (Snedecor and Cochran 1967, p. 159), in this case $y = 5$. Alternatively, we carried out binary logistic regression to calculate the $LE_{50}$ and its confidence interval (Systat ver. 8.0, SPSS), treating individual animals instead of dishes as independent experimental units.

Photoprotective compounds—Carotenoids and mycosporine-like amino acids (MAAs) were extracted from field-collected and laboratory-raised animals. Live animals were stranded on a filter membrane, then sorted into duplicate
samples of 10–50 individuals from laboratory cultures or 100–200 individuals from Lake Giles collections. One sample was dropped directly into 1.5 ml of 100% ethanol for carotenoid extraction (24 h at −5°C). Bulk carotenoids were estimated from spectrophotometric absorbance at the blue absorption maximum and are quantified, hypothetically, as β-carotene: carotenoids (μg mg−1) = 1 × 104 × [OD340/2,620] × [v/w], where v is extract volume (ml), w is total dry mass (mg) estimated from the weight of animals dried for the MAA analysis, OD340 is the optical density at 450 nm (1-cm cuvette), and 2,620 is the absorption coefficient at 450 nm for a 1% (wt:vol) solution of β-carotene (Britton 1995). For MAAs, copepods were counted out live onto pre-tared squares (5 × 5 mm) cut from microscope cover glass, killed with a fractional drop of ethanol, then frozen, freeze-dried, and weighed (Cahn Electrobalance). Extraction was in tightly capped vials containing 0.75–1.5 ml of 25% aqueous methanol (24–48 h at −5°C, sonicated before analysis). *Peridinium* cultures were filtered (Whatman GFC), frozen, then extracted for 24 h in 25% methanol with an additional step of heating for 2 h at 45°C (Tartarotti and Sommeruga 2002). The heating step substantially increased extraction from the alga (ca. 2×), but not the copepod. We have not investigated the possibility that heating causes loss of usujirene (Malcolm Schick pers. comm.). This effect, if present, is obscured by increased extraction upon heating.

MAA extracts were analyzed chromatographically by isocratic high-performance liquid chromatography (HPLC) (Dunlap and Chalker 1986). At Lehigh we used a Shimadzu LC-10AD/SCL-10A chromatograph with SPD-10A-V stopflow scanning spectrophotometric detector (recording 313, 340 nm) and a Brownlee RP 8 column (Spheri 5, 250 × 4.6 mm). For routine analyses the mobile phase was 25% (vol: vol) aqueous methanol with 0.1% acetic acid, at 0.8 ml mm−1. The system was calibrated with *Porphyra*-334 from a concentrated extract of *Porphyra tenera* (commercial dried nori). *Porphyra*-334 peaks were collected from multiple injections of the concentrated extract, combined, diluted to 80% methanol, and quantified spectrophotometrically using a molar extinction coefficient of 42,300 at 334 nm (Karentz 2001). Serial dilutions in 25% aqueous methanol then were injected (20 μl Rhodyne loop and valve) to establish a general calibration factor (area units per μmol) and to confirm the linear response. For other identified MAAs the general calibration factor was multiplied by the ratio of molar extinction coefficient to that of *porphyra*-334 (coefficients tabulated in Karentz 2001).

In the absence of commercially available standards, MAAs were identified by absorption spectra and cochromatography with previously characterized MAAs from tissues of several marine organisms (shinorine—*Porphyra yezoensis*; mycosporine-glycine, asterina-330, palythene—*Palythoa tuberculosa*; palythene—*Devalerae ramentacea*). This work was carried out by R. Sommeruga at the Institute of Zoology and Limnology in Innsbruck, using a Dionex HPLC system including UVD340S diode array detector, chromagram processing software, and a Phenomenex C8 column (Phensphere 5-μm pore size, 250 × 4.6 mm). Two samples were characterized: (1) a bulk diaptomid sample from Lake Giles (September 1995) used over several years as a routine standard at Lehigh and (2) the *P. inconspicuum* from these experiments. The compounds listed above were identified in one or both of the samples and shown to elute with the reference material in one or both of 25% methanol and 55% methanol as mobile phase (each with 0.1% acetic acid). In addition, usujirene was identified tentatively by its absorption maximum and close tracking of its isomer palythene in the chromatograms. These six commonly reported MAAs (Karentz 2001) include all of the principal compounds in *Leptodiaptomus* and *Peridinium*. The bulk diaptomid sample assayed in Innsbruck was cochromatographed with samples from Lake Giles (2001–2002) and the experimentally fed animals to confirm identity of reported MAAs. Molar concentrations were converted to mass units using molecular weights implicit in published MAA chemical structures (Karentz 2001; Shick and Dunlap 2002).

The experiments—Experiments were of two types: (1) those examining UV tolerance of copepods raised under different diet/UVR conditions. In all cases late-stage nauplii and copepodids were raised 3–5 weeks under treatment conditions, into adulthood. UV tolerance experiments concluded with phototrans exposures. Unless noted otherwise, UVR treatments during growth used the medium (17 cm) position on the light-table (Table 1), and *Peridinium* comprised 0.2–0.5 (proportion) of total algal biovolume. As a convention in designating growth treatments in the text, “Cry” designates a *Cryptomonas*-only diet and “Per” designates any diet containing *Peridinium* (in proportion 0.1–1.0 *Peridinium* in admixture with *Cryptomonas*). Irradiance is indicated as “PAR” (PAR only) or “UVR” (UVR plus PAR).

**MAA uptake and retention**—Three experiments were performed. (1) **Maximal MAA uptake** was measured in progeny (first and second generation) of copepods collected in November 2002. These were raised under highest UVR (10-cm position) and fed *Peridinium* without *Cryptomonas*, with the aim of maximizing MAA ingestion. (2) **The dietary availability experiment** examined MAA content in adults raised on five different proportions of *Peridinium* (0–0.3 by biovolume) at fixed UVR (17-cm position). At the highest proportion of *Peridinium* two additional treatments were tested: PAR only (under UV-absorbing OP2 acrylic, λ_most = 400 nm) and enhanced UV-B (borosilicate cover replaced with UV-transparent polyethylene film, transmission 57–83% over the range 280–400 nm). For each dietary treatment, aliquots of the daily food additions were composited in a bottle containing acid Lugol’s solution for later counts and biovolume calculations. Food was added near the end of the light period to assure that most cells were consumed before additional growth could occur. At the end (22 d), MAAs were determined on dried, weighed animals and on three samples of the *Peridinium* cultures. Cell biovolumes were calculated for the *Peridinium* cultures (microscopy at ×1,000) to establish MAA content per unit biovolume. Algae in the preserved samples were counted and sized, enabling calculation of cumulative diet (biovolume, MAA) offered per individual copepod. (3) **The MAA retention experiment** investigated MAA losses under different light
conditions. Animals raised on mixed diet (Peridinium 0.5 by biovolume) under UVR + PAR were switched as adults to Cryptomonas-only diet and incubated under UVR + PAR, PAR-only, or in the dark (two replicate dishes each). MAA content was determined after 9 and 16 days.

UV tolerance experiments—Five phototron experiments were run. Copepods were raised on the light table for 3–5 weeks, into adulthood, before exposure to UVR in the phototron. This growth period constitutes the acclimation period. The 17-cm position under UVA-340 lamps was used, except for the 34-cm position in experiment 2. Diet was 0.2–0.3 (proportion) Peridinium except 0.5 in experiment 5. The first experiment was a range-finding test for UVR exposures and is not presented. Two experiments (2, 5) included a comparison of +PR versus −PR phototron treatments for both Cry/PAR and Per/UVR growth treatments. The remaining two experiments (3, 4) compared three growth treatments: Cry/PAR, Cry/UVR, and Per/UVR, but only for +PR.

Two ancillary experiments used extra animals raised along with those used in phototron experiments. The light-table UVR toxicity experiment investigated the potential lethality of irradiance from the UVA-340 lamps. Non–UV acclimated animals were used (Cry/PAR treatment from experiment 4). Twelve animals were placed in each of 12 small Petrie dishes allocated among three irradiance treatments: (1) borosilicate lid under UV-absorbing acrylic (the Cry/PAR prior growth condition), (2) borosilicate lid alone (the Per/UVR growth irradiance), and (3) quartz lid (passing full UV-B component of the UVA-340 lamps). Cultures were fed Cryptomonas and checked daily for 11 d.

The natural sunlight exposure experiment tested the UV-B tolerance of laboratory-raised animals using the natural irradiance of Lake Giles. Extra animals from the Cry/PAR and Per/UVR cultures used for phototron experiment 5 were placed in small polyethylene bags (12 animals in 150 ml lake water) with cultured Ankistrodesmus as food. This green alga is UV resistant, does not produce MAAs, and is eaten by L. minutus though it is not an optimal diet. The bags were inserted into acrylic tubes defining three irradiance treatments (see Morris and Hargreaves 1997): (1) UV-transparent cast acrylic (“UV-B” treatment, including UV-A and PAR), (2) the same acrylic coated with polyester film (0.05 mm DuPont Mylar D) to remove UV-B (“UV-A” treatment, including PAR), and (3) an extruded acrylic with 50% transmission cutoff at 380 nm (“PAR” treatment). Tubes were suspended in Lake Giles at 0.5 m (60% of surface 320-nm UV-B, 23°C) for four partly sunny days (12–16 July 2002). Bags then were removed, opened, Cryptomonas was added, and survival was assessed after three additional days in darkness.

Results

Seasonal pattern of MAAs and carotenoids—Copepods from Lake Giles displayed different patterns for content of MAAs compared with carotenoids (Fig. 2). The orange animals collected in spring, when MAAs were lowest, had the highest carotenoid content. MAAs peaked in middle-to-late summer when animals were nearly colorless. Spectropho-

metric scans of ethanol extracts from lake-collected as well as laboratory-raised animals (Fig. 3) always displayed the same two-toothed peak in the carotenoid absorption region (453, 478 nm). The scans often showed a peak near 330 nm that corresponds to the MAA absorption region.

Peridinium as a source of MAAs—This dinoflagellate was selected as a MAA source because it is nearly the same size as the cryptomonad (Table 2) and proved to be readily ingested and digested. In the maximal MAA uptake experiment, first and second generation progeny of field-collected animals fed only Peridinium grew well, accumulating total MAA to ca. 0.45% of dry weight. The Peridinium itself contained ca. 0.8% MAA (Table 2, calculating dry weight as 2 × organic C). The six MAAs extracted from the adult copepods occurred in the alga (Fig. 4), although the alga had very little asterina-330 (AS). Quantitatively, the predominant copepod MAAs, palythine (PI) and shinorine (SH), were preferentially accumulated compared with longer wavelength absorbing palythene (PE) and usujirene (US). The alga contained two unknowns—possibly MAAs—with absorption maxima at 332 nm and 318 nm that were never detected in field-collected or laboratory-raised copepods. The 332-nm compound coeluted with PI in 25% methanol in our routine analyses but was separable in 55% methanol. Reanalyses in 55% MeOH were performed on a small subset of Peridinium and copepod samples.
Absorbance of 1-ml ethanol extracts of copepods showing carotenoid and MAA peaks. Animals collected from spring through summer 2002 in Lake Giles are compared with animals raised under different food/irradiance conditions. Diets: Cryptomonas only (Cry) or Peridinium plus Cryptomonas (Per + Cry). Irradiance: PAR-only or UVR + PAR ("low, high UV").

MAA acquisition and retention by copepods—The dietary availability experiment confirmed that MAA accumulation requires a dietary source of MAAs. When Peridinium was offered at only 2% of total algal biovolume, amounting to 5 ng cumulative dietary MAA per copepod over 4 weeks, the net accumulation similarly was ca. 5 ng MAA per copepod (Fig. 5). Total accumulation was much higher (23 ng MAA per copepod) when Peridinium made up 20% of the diet. At the end of the experiment copepods weighed ca. 3.5 μg, so the net uptake of 23 ng MAA per copepod approached 0.7% of dry weight. The background level of 4 ng MAA in animals fed only Cryptomonas represents MAAs carried over from Peridinium consumption as nauplii and young copepods, before the experiment was set up. The pattern of accumulation indicates highly efficient acquisition at limiting MAA availability, with a leveling off at high availability (as suggested by the hyperbolic curve in Fig. 5). Animals raised under polyethylene film instead of the borosilicate lids, and thus exposed to extra UV-B from the UVA-340 lamps, survived well, without accumulating noticeably more MAA. Animals completely protected from UVR ("PAR only") accumulated half as much MAA as those in the UVR series (one-way analysis of variance [ANOVA]; p = 0.003, df = 1). Accumulation of total MAA reached 0.66% dry weight in this experiment, exceeding the uptake when the diet was pure Peridinium (total MAA content 0.45% dry weight).

The composition of MAAs in the copepods is compared with the composition in their diet for the 5-ng dietary MAA treatment (Fig. 6). Once again, PI and SH were accumulated.

Table 2. Algae cultured as food for laboratory copepods. Values are means per cell (±SD) from three cultures used in the mycosporine-like amino acid (MAA) acquisition experiment. Cultures were in transition to stationary state.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cryptomonas reflexa</th>
<th>Peridinium inconspicuaum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>White Acre Pond, Pennsylvania</td>
<td>UTEX LB 2255</td>
</tr>
<tr>
<td>Medium</td>
<td>modified MBL*</td>
<td>Soil water + pea</td>
</tr>
<tr>
<td>Biovolume (μm³)</td>
<td>800 ± 150</td>
<td>720 ± 34</td>
</tr>
<tr>
<td>C (pg)</td>
<td>480 ± 130</td>
<td>520 ± 190</td>
</tr>
<tr>
<td>N (pg)</td>
<td>53 ± 17</td>
<td>45 ± 16</td>
</tr>
<tr>
<td>MAA (pg)</td>
<td>none</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

* Williamson and Butler (1987).
UV acclimation in a copepod

Copepodids were raised 4 weeks, into adulthood, under UVR + PAR or PAR only. Diet was a variable proportion of *Peridinium* (0–0.2) with *Cryptomonas* in a constant total biovolume. Culture lids were borosilicate except polyethylene film in one treatment (“PE”). Acquisition is plotted as a function of cumulative dietary MAA consumed per copepod over 4 weeks ($n = 2$ duplicate subcultures). Final copepod mass averaged 3.5 $\mu$g dry weight.

preferentially over PE and US. Accumulations of PI and SH were approximately 100% of the cumulative amounts offered in their diet. This comparison assumes that chemical extraction of MAAs from *Peridinium* was complete—an uncertain issue for further testing—and ignores possible interconversion from the less accumulated MAAs. Figure 6 also shows the MAA composition of animals collected from Lake Giles. *Peridinium* in the laboratory evidently provided a fair simulation of the natural dietary MAAs, leading to the same suite of compounds and the same predominance of PI and SH.

The MAA retention experiment demonstrated similar declines of total MAA under the three irradiance treatments (Fig. 7). The overall exponential fit corresponds to a half-life of ca. 23 d. The decline in concentration was principally a loss, rather than dilution by increasing copepod mass, since mass did not change appreciably over the 16-d experiment. In contrast, decline of the most abundant MAA, PI, differed significantly among treatments (two-way ANOVA using data from days 9 and 16; $p < 0.01$). Under sustained UVR, PI was more highly conserved than total MAA, and thus more conserved than the other principal MAAs, SH and PE.

The light-table UVR toxicity experiment showed that multiday exposure to UVA-340 lamps at the 17-cm position was lethal to nonacclimated animals. (These results are summarized here, but not presented in detail.) Nonacclimated Cry/PAR adults started dying after 3 d under the borosilicate lids, with 87% mortality by day 11. Under quartz lids, which transmitted 50% more UV-B (280–320 nm), mortality
reached 100% by day 3. Controls under UV-absorbing acrylic were all alive on day 11. Therefore, radiation from the UVA-340 lamps constitutes an environmental stress to which the animals can become acclimated, if raised initially at intermediate intensities (e.g., the Per/UVR treatment).

UV-B tolerance in the phototron—Copepods raised for phototron experiments were either preacclimated to UVR during growth (Per/UVR, Cry/UVR) treatments or not (Cry/PAR). In the phototron, copepods potentially received an acute 12-h UV-B exposure. The maximum UV-B intensity in the phototron approached the UV-B wattage of full sunlight (Table 1) and was biased toward shorter, more damaging wavelengths (Fig. 1). The course of survival following UVR exposure in the phototron indicates relative degree of UV tolerance. Differences among growth treatments are illustrated in Fig. 8. These results were corrected, day-by-day, for natural baseline mortality. After 5 d of post-exposure incubation in darkness, there were obvious differences in mortality related to both diet and irradiance. In the 95 kJ m⁻² exposure, the Per/UVR treatment was significantly more tolerant than the Cry/UVR treatment (t-test, p < 0.001). In the 54 kJ m⁻² exposure, the Cry/UVR treatment was more tolerant than the Cry/PAR treatment (p < 0.001).

The calculation of LE₅₀ with 95% confidence intervals is illustrated in Fig. 9, which contrasts Cry/PAR and Per/UVR cultures, both with and without PR radiation. The log/logit transformations linearize what is generally a sigmoid response. Only points within or immediately bracketing the region of partial mortality were used in the regression. Photorepair contributed importantly to UV-B tolerance in both the nonacclimated Cry/PAR treatment (3.5-fold increase in LE₅₀, or +41 kJ m⁻²) and the Per/UVR treatment (threefold increase, or +85 kJ m⁻²). The UV-acclimated, MAA-rich Per/UVR treatment was more tolerant than Cry/PAR, both without PR (3.5-fold, or +33 kJ m⁻²) and with PR (2.5-fold, or +77 kJ m⁻²).

Alternate confidence intervals calculated using binary logistic regression (Fig. 10, thicker error bars) are smaller than those calculated by treating dishes as primary experimental units, owing to the greater replication. The validity of statistics from binary logistic regression in this case would depend on an absence of dish-to-dish effects, which cannot generally be assured. Nevertheless, chi-square tests for dish-to-dish heterogeneity were negative (single classifications with equal expectations—Snedecor and Cochran 1967, p. 231). For all 18 exposure treatments of 4–5 dishes (ca. 10 animals per dish) that showed an intermediate response (average mortality within the range 20–80%), p exceeded 0.15 in all cases and was equally distributed around p = 0.5.

Results of the four principal phototron experiments are summarized in Fig. 10, comparing contents of carotenoids and MAAs as well as LE₅₀. Note that the Per/UVR treatment in experiment 2 was raised at lower UVR intensity than in the other experiments, consistent with the lower MAA

Fig. 8. Phototron experiment 3. Survival of L. minutus following 12-h UV-B exposure (with photorepair radiation). Copepods were raised under three food-irradiance treatments: Cryptomonas (Cry) in PAR, Cryptomonas in UVR + PAR, or Peridinium plus Cryptomonas (Per + Cry) in UVR + PAR. Each growth treatment was assayed at two of three UVR exposures. Values are means ± SE for n = 5 dishes of ca. 10 animals each.

Fig. 9. Phototron experiment 5. Survival at day 5 following UV-B exposure, with and without photorepair radiation (6 PR). (a) Means with SD of four to five dishes, linear scales. (b) Transformed data used to calculate LE₅₀ (x) and 95% confidence intervals. Copepods were raised in UVR + PAR on a diet of Peridinium plus Cryptomonas (Per, diamonds) or in PAR with only Cryptomonas (Cry, triangles).

Fig. 10. Log10 (kJ m⁻² 12 h⁻¹) vs. UVR exposure (kJ m⁻² 12 h⁻¹) for all four experiments with 95% CI's for 50% survival.
content and lower LE$_{50}$. MAAs accumulated only when Peridinium was included in the diet. A small amount of MAAs in the Cry/Par treatment of experiment 5 represents carryover from feeding by young copepodids before the treatments were segregated. In general, the semireplicate phototron experiments (2, 5 and 3, 4) gave consistent results. The Per/UV preacclimation during growth on the light-table increased UV tolerance by ca. 2.5-fold over the Cry/Par treatment. The Cry/UV treatment was intermediate between the Per/UV and Cry/Par treatments.

One surprising result was the high accumulation of carotenoids under UVR when Cryptomonas alone was the food source, but not when Peridinium made up part (20–50%) of the diet. Cry/UV copepods became strongly orange, whereas the MAA-containing animals were only slightly yellowish, and sometimes as pale as Cry/Par animals. Scans of ethanol extracts showed a strong similarity between Cryptomonas-derived carotenoids and carotenoids accumulated under natural conditions in Lake Giles (Fig. 3).

Response to natural sunlight—UV-B was high enough at a depth of 0.5 m in Lake Giles to significantly decrease survival of Cry/Par animals compared with the Per/UV animals ($t$-test, $p < 0.01$; Fig. 11). The mortality was caused by UV-B. Thus, laboratory-acclimated Per/UV animals seem capable of tolerating average near-surface irradiance conditions in nature. UV-B tolerance might be less complete under conditions of slightly greater exposure, for instance, precisely at the lake surface, under completely cloudless skies, or over a longer incubation.

Discussion

MAA acquisition and retention—This is one of the first studies to quantify the acquisition of mycosporine-like amino acids by a freshwater zooplankter and to suggest that UVR exposure enhances uptake. L. minutus raised under PAR (at 5–10% of full sunlight levels) did sequester MAAs, but adding UVR to the growth irradiance doubled the accumulation from the same diet (Fig. 5). Previous studies with sea urchins and other marine invertebrates have demonstrated MAA accumulation from benthic algae (Carroll and Shick 1996; Adams and Shick 1996; subsequent studies cited in Shick and Dunlap 2002). Adams et al. (2001) found that the presence or absence of UVR did not affect the amount of MAAs sequestered in ovaries of adult sea urchins. These MAAs protected the subsequent planktonic larval stage (Adams and Shick 2001), perhaps explaining the insensitivity of uptake to irradiance conditions experienced by the benthic adults. Helbling et al. (2002) recently linked MAA accumulation by another freshwater calanoid copepod, Boeckella, to UVR exposure during growth, and demonstrated enhanced tolerance of both UV-B and UV-A. Tartarotti et al. (2001) established a strongly positive multilake correlation between MAA concentration in a cyclopoid copepod, Cyclops, and its UVR environment. It is generally unclear, however, whether such relationships represent stress responses of the consumers or merely reflect MAA availability in food organisms (Shick and Dunlap 2002). Our results show that MAA uptake by consumers living under UVR stress can be highly efficient at low MAA availability in the diet (ap-
planktonic crustacean Dunlap 2002; Banaszak 2003). The suite of MAAs taken up
not be synthesized by animals (Karentz 2001; Shick and
their diet supports the general presumption that MAAs can-
UVR stress unless the compounds were accessible through
much by dietary availability as by UVR stress. Not all algae or
sea urchin Strongylocentrotus (Adams and Shick 1996,
results have been reported for marine invertebrates such as the
environment, although the proportions differed. Similar
parently approaching 100%; Figs. 5, 6). Not all algae or
MAA content of consumers is likely to be controlled as
(Williamson et al. 2001), the full 12-h exposure (ca.
340 lamps did not significantly affect the loss rate of MAA
UV-A lamp used in our experiments actually reduced MAA
Evidently those free-living animals were not accli-
UV-B region. Exposure to UVR from the UVA-
resultant UVR screening in the
resultant absorption MAAs palythine and shinorine than
polyethylene and usujirene. Resultant UVR screening in the
300–340 nm region appreciably overlapped the output of the
UVB-312, as well as the UVA-340 lamps (Fig. 1), assuming
in vivo absorption spectra were similar to those of extracted
Although shinorine has peak absorbance in the
TIME TO EURYTHROCYTES
were the same as the MAA content
observed mortality. True long-term tolerance levels for
surviving to day 5 may be reproducibly compromised if not
Karanas et al. 1979). The maximum daily
exposure tolerated over multiday treatments would likely
be even lower, although repair processes—including the
repair measured in this study—can prevent chronically low
exposures from accumulating to a lethal dose (Grad et al.

The in-lake experiment confirmed that acclimation under the
UVA-340 lamps, with attendant MAA accumulation, protected against UV-B in natural sunlight. This is not surprising, since the MAAs present in L. minutus should be even more effective against natural solar radiation—and the
UVA-340 growth irradiance—than against radiation from

Phototron as acute toxicity assay—Our phototron ap-
approach combines acute UV-B stress with a statistically con-
venient criterion for tolerance (LE_50) assessed after an arbi-
trary 5-d dark interval for mortal damage to translate into
observed mortality. True long-term tolerance levels for
healthy, reproducing populations must be substantially low-
for, example, than the 125 kJ m^-2 LE_50 (with PR) com-
puted for Per/UVR-raised L. minutus (Fig. 10). Animals sur-
viving to day 5 may be reproducibly compromised if not
actually moribund (Karanas et al. 1979). The maximum daily
daily exposure tolerated over multiday treatments would likely
be even lower, although repair processes—including the
repair measured in this study—can prevent chronically low
exposures from accumulating to a lethal dose (Grad et al.

The 23-d half-life for MAA retention is potentially long
enough to sustain photoprotection through intervals of diet-
ary scarcity in summer. Exposure to UVR from the UVA-
340 lamps did not significantly affect the loss rate of MAA
as a whole from animals abruptly deprived of their MAA
source. Evidently MAAs were not being rapidly degraded
by the growth irradiance, consistent with the apparently high
efficiency of net accumulation over weeks of growth. Adams
and Shick (2001) found that UVR from the same type of
UV-A lamp used in our experiments actually reduced MAA
losses from sea urchin larvae. In contrast, the more intense
UVR of natural sunlight accelerated losses of MAAs from
another freshwater copepod (Helbling et al. 2002). The
MAA content of laboratory-raised L. minutus reached 0.4–
0.7% of dry weight, exceeding levels encountered in Lake
Giles in summer (0.1–0.3%), but still below maximum levels
cited for copepods from high-UVR environments (1–3% dry
weight; Tartarotti et al. 2001).

Increased UV-B tolerance—The LE_50 of preacclimated
Per/UVR animals was 2.5 times that of nonacclimated Cry/
PAR animals. This can be characterized more specifically as
greater UV-B tolerance, even though the phototron UVB-
312 lamps also produce considerable UV-A (Fig. 1). Spectral
weighting functions developed for copepods (Kouwenberg
et al. 1999; Tartarotti et al. 2000; Helbling et al. 2002) and
other organisms assign rapidly increasing damage per unit
energy to progressively shorter wavelengths in the 280–320
nm range, presumably reflecting damage to DNA. Based on
a similar weighting function developed for Daphnia puli-
caria (Williamson et al. 2001), the full 12-h exposure (ca.
160 kJ m^-2) from the three-lamp phototron used in our ex-
periments would have three times the damaging potential of
a completely sunny July day at Lake Giles. Ninety percent
of the damage would originate at wavelengths below 320
nm, or in the UV-B region.

Williamson et al. (1994) previously found that natural UV-
B caused significant mortality in lake-collected L. minutus,
when animals were exposed in polyethylene bags under con-
ditions similar to those used in our natural sunlight experi-
ment. Evidently those free-living animals were not accli-
ated to near-surface irradiance, through some combination
of lower UVR exposure during growth (daytime avoidance
of surface waters?) and limited MAA availability in their
diet. The UV-A of bright sunlight can also affect copepod
survival (Zagarese et al. 1997a; Tartarotti et al. 2000; Hel-
bling et al. 2002), but UV-A sensitivity was not evident
when L. minutus was exposed to partly sunny weather in
Lake Giles (Fig. 11, also Williamson et al. 1994).

The Per/UVR preacclimation arguably increased UV-B
tolerance in the phototron by similar multiplicative factors
(ca. 2.5-fold) in the presence and absence of photorepair
radiation. Constancy of this factor would be consistent with
the UV-screening function of MAAs. This interpretation ac-
counts for the greater additive benefit of PR to the Per/UVR
versus Cry/PAR treatments (+85 vs. +35 kJ m^-2, respec-
tively), without needing to invoke upregulation of photore-
pair in UVR-raised animals. The Per/UVR acclimation thus
increased UVB tolerance by a magnitude similar to that pro-
vided by photorepair alone (2–4 fold). This comparison as-
sumes that photorepair was not significantly underestimated
in the phototron, where the longer UVR from the UV-B
lamps could have stimulated some photorepair, and where
the shortest UVR from the photorepair lamps may have
caused some damage, offsetting part of the photorepair.
The magnitude of such an underestimation is believed to be small
in the case of L. minutus but cannot be evaluated with ex-
isting data. However, this issue does not affect conclusions
about the effectiveness of the Per/UVR acclimation in
increasing UV-B tolerance.
the UVB-312 lamps used in the phototron. Phototron irradiance is shifted to shorter wavelengths (Fig. 1) that extend below the effective in vitro absorption of the principal MAAs palythine (wavelength range for 50% of maximum absorbance: 296–332 nm) and shinorine (310–344 nm).

Alternate strategies? MAAs versus carotenoids—The specific contribution of MAAs to the higher UV-B tolerance of Per/UVR animals was not quantitatively established by this study. The comparison of Per/UVR to Cry/PAR animals (2.5-fold increase in LE50) may overstate the MAA effect if preacclimation to UVR stimulated DNA repair or other protective mechanisms independently of MAA accumulation (for example, various antioxidant responses—Vega and Pizarro 2000; Hessen 2003). On the other hand, comparison with the Cry/UVR treatment (only 1.5-fold increase in LE50) will underestimate the effect if, as seems likely, the observed carotenoid accumulation in Cry/UVR animals represents an alternative photoprotection strategy.

The LE50 for orange Cry/UVR animals in this study was intermediate between those of paler Cry/PAR and Per/UVR animals (Fig. 10), suggesting that carotenoid accumulation might have offered some UV-B photoprotection but less than MAAs. Carotenoids should be much less effective than MAAs in screening out UVR, based on maximal carotenoid absorbance in the blue region of the spectrum. However, carotenoids also can be potent intracellular antioxidants, quenching excited oxygen and neutralizing free radicals (Edge et al. 1997), and thereby blocking some of the chemical damage to DNA and other molecules induced by UV-B as well as UV-A radiation. Most MAAs function directly as sunscreens, dissipating absorbed UVR as heat without producing harmful radicals or excited oxygen (Shick and Dunlap 2002). One MAA present at low concentration in L. minutus, mycosporine glyaine, also has significant antioxidant activity in vitro (Shick and Dunlap 2002; Suh et al. 2003).

Enhanced retention of dietary carotenoids by strongly pigmented copepods generally has been interpreted as a response, at least in part, to high irradiance (Hairston 1976; Luceke and O’Brien 1981; Byron 1982). Some studies link carotenoids specifically to increased UV tolerance (Ringelberg et al. 1984; Hansson 2004), although Zagarese et al. (1997a) found that photorepair accounted for the relatively high UV-B tolerance in red Boeckella gibbosa. In our study, UVR-stressed L. minutus apparently switched from carotenoid accumulation to MAA accumulation when dietary MAA was available. This was surprising, since MAAs cooccur with high levels of carotenoids in the bright red copepods of some high-UVR lakes (Sommaruga and Garcia-Pichel 1999; Tartarotti et al. 1999). Indeed, unanalyzed MAAs may have contributed to the photoprotection claimed for carotenoids in earlier studies of red copepods. Substituting Peridinium for 20–30% of the Cryptomonas in the mixed diet conceivably reduced availability of usable carotenoids, but seemingly not enough to account for the drastic reduction in carotenoid accumulation measured in Cry/UVR versus Per/UVR animals (Fig. 10).

Many colored freshwater and marine copepods use the keto-carotenoid astaxanthin and its esters as the principal pigments (Hairston 1976; Bandaranayake and Gentien 1982; Ringelberg et al. 1984). In L. minutus, however, keto-carotenoid does not seem to predominate. The two-peaked absorption spectra (453, 478 nm; Fig. 3) are more indicative of β-carotene or hydroxy-xanthophylls than astaxanthin, which has a single broad peak near 478 nm (Britton 1995; copepod extracts of Tartarotti et al. 2001 and Hessen 2003). Regardless of the specific compounds present, the carotenoid content in UVR-exposed L. minutus reached 0.4–1% dry weight, as in other red, presumably astaxanthin-containing, copepods (Tartarotti et al. 1999).

Ecological significance—This study illustrates how opportunistic use of dietary compounds can be an important component of physiological acclimation to ambient UVR. The 2.5-fold increase in LE50 associated with MAA accumulation in L. minutus brings the animals close to the full tolerance of lake surface UVR conditions seen in highly pigmented copepods from UV-transparent lakes (Zagarese et al. 1997b; Rocco et al. 2002). The broad functional analogy of MAAs with carotenoids in aquatic food webs raises familiar issues from carotenoid-oriented studies. These include sunscreen versus antioxidant roles, availability in different food organisms, selective uptake and metabolic modification by different consumers, allocation into reproductive stages, and effects of environmental factors such as temperature or light/UVR. Both classes of compounds can reach levels near 1% of dry weight in copepods, producing similar in vitro absorbance at the respective wavelength maxima. For example, 1% MAA (as palythine) would absorb only 25% less at 320 nm than does 1% carotenoid (as astaxanthin) at 478 nm. Unlike blue-light absorbing carotenoids, however, MAAs might not increase the risk of predation by visually feeding vertebrates, which preferentially take heavily pigmented copepods (Hairston 1979a; Luceke and O’Brien 1981; Hansson 2004). Some fish have visual photoreceptors that function into the longer UV-A wavelengths (Leech and Johnsen 2003), but there is little evidence for effective vision below 350 nm, or into the absorbance band of the MAAs preferentially accumulated by L. minutus. Possibly the 360-nm absorbing MAAs are not retained in order to avoid increasing the apparency of copepods to UV-detecting predators.

Seasonal cycles of carotenoid like that observed in Lake Giles animals—high in spring, low in summer (Fig. 2)—have been interpreted as resulting from selective predation against strongly pigmented animals (Hairston 1979a,b). Hansson (2004) further argues that carotenoid pigmentation responds to a seasonally changing tradeoff between threats from UVR and predation, with individual copepods down-regulating carotenoid accumulation in the presence of zooplanktovorous fish. A new insight from L. minutus is that carotenoid accumulation may not respond positively to UVR if MAAs are available in the diet. Switching to an alternate MAA-based photoprotective strategy might contribute to the inverse spring-to-summer pattern of MAAs and carotenoids in Lake Giles, where fish predation likely is also important. Sequestration of dietary MAAs would then constitute an evolutionary adaptation securing UV protection while minimizing predation, but it would be constrained by more restricted availability of MAAs compared with carotenoids among food items. Of course, this scenario needs to be con-
firmed using other dietary sources of MAAs and carotenoids. Conceivably, carotenoid uptake in *L. minutus* could have been incidentally blocked by some constituent of the particular dinoflagellate used as a source of MAAs.

Finally, MAA use by copepods may differentiate them ecologically from co-occurring and potentially competing populations of cladocerans, which seem to lack MAAs in Lake Giles (A. Persaud, R. Moeller, and C. Williamson unpubl. data) and elsewhere (Tartarotti et al. 2001; Goncalves et al. 2002; Hessen 2003).

**References**


Received: 16 April 2004
Accepted: 7 October 2004
Amended: 15 November 2004