Belg. J. Zool., 143(2) : 106-118 July 2013

Hurdles in investigating UVB damage in the putative ancient asexual *Darwinula stevensoni* (Ostracoda, Crustacea)

Lynn Van den Broecke[1,2], Jacques Vanfleteren[2], Koen Martens[1,2] & Isa Schön [1,3,*]

[1] Royal Belgian Institute of Natural Sciences, Freshwater Biology, Vautierstraat 29, 1000 Brussels, Belgium.
[2] University of Ghent, Biology, K.L. Ledeganckstraat 35, 9000 Gent, Belgium.
[3] University of Hasselt, Biology, Universitaire Campus Gebouw D, B-3590 Diepenbeek, Belgium.
* Corresponding author: isa.schoen@naturalsciences.be

ABSTRACT. Ostracoda or mussel-shrimps are small, bivalved Crustacea. Because of their excellent fossil record and their broad variety of reproductive modes, ostracods are of great interest as a model group in ecological and evolutionary research. Here, we investigated damage and repair from one of the most important biological mutagens, namely UVB radiation, in the putative ancient asexual ostracod *Darwinula stevensoni* from Belgium. We applied three different methods: the Polymerase Inhibition (PI) assay, Enzyme-Linked Immuno Sorbent Assay (ELISA) and dot blot. All three techniques were unsuccessful in quantifying UVB damage in *D. stevensoni*. Previous experiments have revealed that the valves of *D. stevensoni* provide an average UVB protection of approximate 60%. Thus, UVB damage could be too little to make quantitative experiments work. Additionally, variation between individual ostracods due to season and age most likely contributed further to the failure of the three used experimental approaches to quantify damage. In a second experiment, we investigated the influence of temperature on survival of *D. stevensoni* during UVB exposure. The estimated relative lethal UVB dose at 4°C was with 50 kJ/m^2, significantly lower than at room temperature, with 130 kJ/m^2. This could either indicate lack of adaptation to low temperatures and/or the presence of metabolic processes active at room temperature protecting against UVB damage in *D. stevensoni*. The latter possibility could also explain why the estimated relative lethal UVB dose of *D. stevensoni* is similar to that of other non-marine ostracods where valves provide around 80% protection, despite the valves of *D. stevensoni* providing less protection. If such metabolic processes can repair UVB damage quickly, this may provide an alternative explanation why we could not quantify UVB damage in *D. stevensoni*.

KEY WORDS: *Darwinula stevensoni*, ancient asexual, DNA repair

INTRODUCTION

UVB (280-315 nm) radiation is one of the most important biological mutagens (Setlow et al., 1993; Rautio & Tartarotti, 2010), inducing the formation of cyclobutane pyrimidine dimers (CPDs) (Sancar & Tang, 1993; Rautio & Tartarotti, 2010), which inhibit DNA transcription and translation (Sancar & Tang, 1993). One way for organisms to remove these CPDs is excision repair (ER), which tends to be common across eukaryotes, but can be energetically costly if more than a single nucleotide requires repair (Sancar & Tang, 1993). Another important process of repair from UVB damage is enzymatic photoreactivation (Jagger & Stafford, 1965). The two enzymes involved in this process both use light energy; CPD-photolyase removes CPDs, while [6-4]-photolyase reverses pyrimidine-[6-4']-pyrimidine photoproducts (Friedberg et al., 1995).

Ostracoda or mussel-shrimps are small, bivalved Crustacea. Ostracods are very common in most surface waters, marine and non-marine, but they also occur in interstitial and even (semi-) terrestrial environments (Martens et al., 2008). These crustaceans are of great interest as a model group in ecological and evolutionary

research, because their calcified valves preserve well as microfossils, especially in lacustrine environments. Their excellent fossil record thus provides real-time frames for evolutionary processes (HOLMES & CHIVAS, 2002). Ostracods can also serve as proxies for climate (HORNE et al., 2012) and ecosystem changes because their fossilized valves allow reconstructing past climatic and environmental conditions (HOLMES & CHIVAS, 2002). In addition, non-marine ostracods are well-suited for investigations on the evolution of sex and parthenogenesis (MARTENS, 1998), because of their variety of reproductive modes. One of the ostracod families, Darwinulidae, reproduces exclusively asexually and is believed to have done so for at least 200 Myr (MARTENS et al., 2003); they thus represent one of the four examples of putative ancient asexuals in the animal kingdom. Karyological and allozyme studies have so far only found evidence for apomictic parthenogenetic reproduction in ostracods (BUTLIN et al., 1998; SCHÖN et al., 1998; SCHÖN & MARTENS, 2003).

SCHÖN et al. (2009) suggested that *D. stevensoni* is the most likely candidate to be a true ancient asexual. No reliable recent or fossil males have been found since at least 25 million years (STRAUB, 1952). Furthermore, this species appears to feature non-meiotic mechanisms such as gene conversion that could homogenize its genome (SCHÖN & MARTENS, 2003; SCHÖN et al., 2009) and possibly, highly efficient DNA repair (SCHÖN & MARTENS, 1998). Previous experiments with UVB (VAN DEN BROECKE et al., 2012) showed a strong correlation between the amount of UVB that is blocked by ostracod valves and the estimated relative lethal UVB doses. Certain ostracod valves blocked 80% and more of UVB radiation, thus providing effective shielding. Pigmented species from temporary habitats were best protected. These species also showed high estimated relative lethal UVB doses of 110 kJ/m^2 to 214 kJ/m^2. *Darwinulia stevensoni* was only protected against about 60% of UVB radiation by its valves, but the estimated relative lethal dose for this species was as high as for the other well-protected ostracods (130 kJ/m^2; VAN DEN BROECKE et al., 2012). These results may indicate that metabolic processes could also be involved in the repair of UVB damage in *D. stevensoni*.

Because of the lack of sufficient genomic data or any Expressed Sequence Tags, we used the following three techniques to quantify DNA repair in *D. stevensoni* after UVB exposure: the Polymerase Inhibition (PI) assay, Enzyme-Linked Immuno Sorbent Assay (ELISA) and dot blots. In an additional experiment, the influence of the temperature on UVB exposure and DNA damage was investigated. It has been suggested that UVB is a more important stressor at colder temperatures because enzymatic processes such as DNA repair mechanisms are slower at lower temperatures (HESSEN, 1996).

MATERIAL AND METHODS

Material

Darwinula stevensoni is common in all kinds of aquatic non-marine habitats, including lakes, rivers and interstitial habitats, freshwater to saline environments and arctic to (sub-) tropical conditions. All darwinulids are brooders and *D. stevensoni* has an average of 11–15 offspring in temperate regions (VAN DONINCK et al., 2003). Darwinulids generally have low fecundity as compared to other ostracods (GEIGER, 1998) and rather long life cycles of up to four years (MCGREGOR, 1969; RANTA, 1979). These features have so far made it impossible to establish synchronized long-term mass cultures as would be needed to test, for example, for maternal effects.

Darwinula stevensoni was collected from 'Hollandersgatkreek' (51° 16' 08'' N, 03° 32' 07'' E; Sint-Laureins, Belgium), where a monoclonal (as identified by the genetic markers COI and *Pgi*) population is known to occur in high densities throughout the whole year. All samples were randomly taken with a 200 µm mesh hand-net and subsequently stored in the laboratory for

acclimatisation as mass cultures in their habitat water at 15°C for one generation (because of the exceptionally long generation time, see above).

The experiments were performed using individuals that had been collected at various times during the course of the year. For the PI-experiments, individuals collected in spring, autumn and winter were used. For the ELISA-experiments, a pilot experiment investigated individuals from autumn, and for the subsequent experiment, ostracods from spring collections were used. For the dot blot experiments, individuals sampled in spring were screened in a pilot study, and samples collected in summer used for the more extensive study.

Methods

UVB exposure

To determine DNA damage as a response to UVB exposure, individual adult females of *Darwinula stevensoni* were randomly taken from the mass cultures. For all experiments with ostracods, six biological replicas were conducted in individual Petri dishes with EPA medium (96mg/L $NaHCO_3$, 60mg/L $CaSO_4.2H_2O$, 123mg/L $MgSO_4.7\ H_2O$, 4mg/L KCl and pH 7.4-7.8) on ice. The ostracods were exposed to UVB light from a 6W Vilber Loumat UV lamp (λ = 312 nm) with an intensity of 650 µW/cm^2 at a distance of approximately 15 cm to the lamp. UV intensity was measured with an UVB radiometer (UVP®). Exposure started with a dose of 1.95kJ/m^2 (= ±5 min), with a maximum of 140.4kJ/m^2 (= ±6 h) to ensure a large range of UV doses during exposures.

Polymerase Inhibition assay

The Polymerase Inhibition (PI) assay exploits the well-reported fact that the polymerase enzyme, which is routinely used in PCR reactions, stops replicating when it encounters a UV-induced adduct like a CPD in the template DNA (Jenkins et al., 2000). Consequently, the DNA segment that bears such damage provides a poor substrate for PCR. This will be reflected as a proportional reduction in the amount of amplified DNA from damaged templates as compared to non-damaged templates. On the other hand, DNA repair of the PCR target DNA segment should be measurable as a restoration in the amount of amplified template after exposure to UVB and subsequent repair.

After UVB exposure, the state of the *D. stevensoni* individuals (alive or dead) was recorded and DNA was extracted with the GeneReleaser standard protocol (Eurogentec). Subsequently, 5µl of DNA was used for PCR amplification.

The PI assay requires a fine-tuned optimisation, which was accomplished prior to testing for UVB damage. Three different genomic regions, an 850bp fragment of the single nuclear copy gene *hsp82*, 600bp of the nuclear multi-copy ITS region and 650bp of the mitochondrial COI gene were amplified by PCR to allow for comparisons of DNA repair and damage between nuclear and mitochondrial DNA and single or multi-copy regions. The following, species-specific primers for *D. stevensoni* were developed from existing sequences (Schön et al., 1998; Schön & Martens, 2003) (hsp82 FORW [TGACTACCTGGAGGAGAGGAA], hsp82 REV [CCAACATCCTCTATTTTTGGC], ITS FORW [TATCGTGAACCGTCTTGTCG], ITS REV [CGAGGTCCGACAGAAAGAAA], COI FORW [TACCTAATCTTAGGGGCCTGA], COI REV [AGGTGTTGGTATAGGATTGGG]).

For *hsp82*, an initial denaturation step at 95°C for 5 min was followed by 41 cycles of 15 s at 94°C, 1 min at 50°C and 1 min at 72°C. PCRs were performed in 25 µl volumes with PCR buffer (Tris·Cl, KCl, $(NH_4)_2\ SO_4$, 7.5 mM $MgSO_4$, bovine serum albumin, Triton® X-100, Factor SB; pH 8.7 (20°C), 1.5 mM dNTPs), 5 µl Q solution (Qiagen), 0.5 U Hifidelity Taq polymerase (Qiagen), 10 pmol of each primer, 7.5 µl RNAse free water and 5 µl of DNA template. The same conditions were used for the other two loci, except that the annealing

temperature for COI was 54°C and only 39 cycles were performed, while the annealing temperature for ITS was 52°C with 35 cycles. Electrophoresis of PCR products was conducted on 1.2 % agarose gels, which were stained with GelRed™ and photographed under UV light. The analyses of the relative intensity of PCR products as indicator for DNA damage were conducted with the program Image J (GIRISH & VIJAYALAKSHMI, 2004).

ELISA

In a follow-up on the PI-assay, we additionally performed ELISA experiments to detect the formation of CPDs. An added value of the ELISA technique is that the whole genome is investigated, employing a more direct detection technique using specific antibodies against CPDs. We conducted two different series of ELISA experiments, one pilot study in autumn and a more extensive study in spring. In the ELISA experiments, we exposed three different kinds of material to UVB: extracted DNA (4 replicates), living and dead *D. stevensoni* individuals (6 replicates each). If the bodies or valves of ostracods provide protection to UVB, extracted DNA should show the highest amount of UVB damage. If metabolic processes are involved in UVB protection, living ostracods should show lower UVB damage and more repair than dead ostracods. Thus, our hypothesis is that UVB damage is lowest in living individuals and is higher in dead ostracods, with the highest damage present in the DNA extractions. DNA was extracted from ostracods using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol. After UVB exposure and DNA extraction, DNA solutions in PBS were prepared with a concentration of 0.2 µg/mL following the protocol of MBL®. The DNA was coated to a microtiter plate covered with protamine sulfate. After overnight incubation, the specific Biotin-F(ab')2 fragment of anti-mouse IgG (H+L) (Zymed, Cat. No.62-6340) monoclonal antibody against CPDs (Cosmo Bio Co., Ltd) was distributed into the wells of the plate. After additional incubation, the first antibody was washed off and a second, enzyme-linked antibody was coated to the wells. As a final step, luminol (SUPERSIGNAL WEST FEMTO; Fisher Scientific) was added. Its light reaction indicates that the antigenes, in our case CPDs, are present, while the strength of the signal is proportional to the concentration of CPDs The strength of the luminol light reaction was measured with a VICTOR™ Light Luminescence Counter (PerkinElmer) and background levels were subtracted.

Dot blot

The third experimental approach included dot blot experiments, which have been successfully used to detect one type of CPDs (thymine dimers) in bacteria, phytoplankton and macroalgae (SINHA et al., 2001). Dot blotting is a simple technique being routinely used in laboratories to identify a known biomolecule in a biological sample. The ease and simplicity of the technique makes dot blotting an ideal diagnostic tool. In our experiments, dot blotting involved almost the same protocol as ELISA and thus the same specific monoclonal antibody against CPDs (Cosmo Bio Co., Ltd) was used. The most important difference to ELISA is the immobilization of DNA on a binding membrane, usually nitrocellulose or polyvinylidene fluoride, instead of a microtiterplate. DNA was extracted from ostracods using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol. For the dot blot, we mainly applied the protocol by SINHA et al. (2001) with some small modifications. After DNA extraction, we blotted 10ng DNA on an Amersham Hybond ECL Nitrocellulose Membrane (GE Healthcare). The visualization of the dot blot occurred with luminol, similarly to the ELISA experiment, but with a different variant (SUPERSIGNAL WEST PICO; Fisher Scientific) being less sensitive. Photos of the nitrocellulose membrane with the dots (Fig. 1) were developed on an Amersham Hyperfilm (GE Healthcare). Analyses of the intensity (darkness) of the dots on the membrane, and thus the relative amount of DNA damage, were conducted with the program Image J

(Girish & Vijayalakshmi, 2004). Animals for the pilot dot blot were sampled in spring, for the more extensive experiment in autumn.

Temperature-dependent experiment

All the UVB exposures for the PI assay, ELISA and dot blot experiments were conducted at room temperature. To examine the influence of the temperature on UVB damage, we also exposed 10 *D. stevensoni* individuals, placed in separated containers, to UVB at 4°C. At the same time, we placed 10 individuals at 4°C and 10 individuals at 25°C as controls without UVB exposure. The status of the animals was checked every 30 minutes.

Statistical analyses

All statistical analyses were conducted with STATISTICA. Data obtained from the different biological replicates were first tested for normality and homoscedasticity. If replicates for each treatment were normally distributed and did not differ in their variance, the average of the replicates was calculated per treatment and used for subsequent One-Way ANOVAs and, where applicable, for post-hoc Tukey HSD tests. Also, the correlation coefficient R and its statistical significance were calculated. For statistically comparing the estimated relative lethal doses of UVB at 4°C and room temperature, we conducted Chi Square tests.

RESULTS

Polymerase Inhibition assay

When the amount of PCR amplicons from living *D. stevensoni* (cultured at 15°C) is plotted against the intensity of UVB exposure, no clear pattern is observed for any of the three screened genetic regions (see Fig. 2 for the

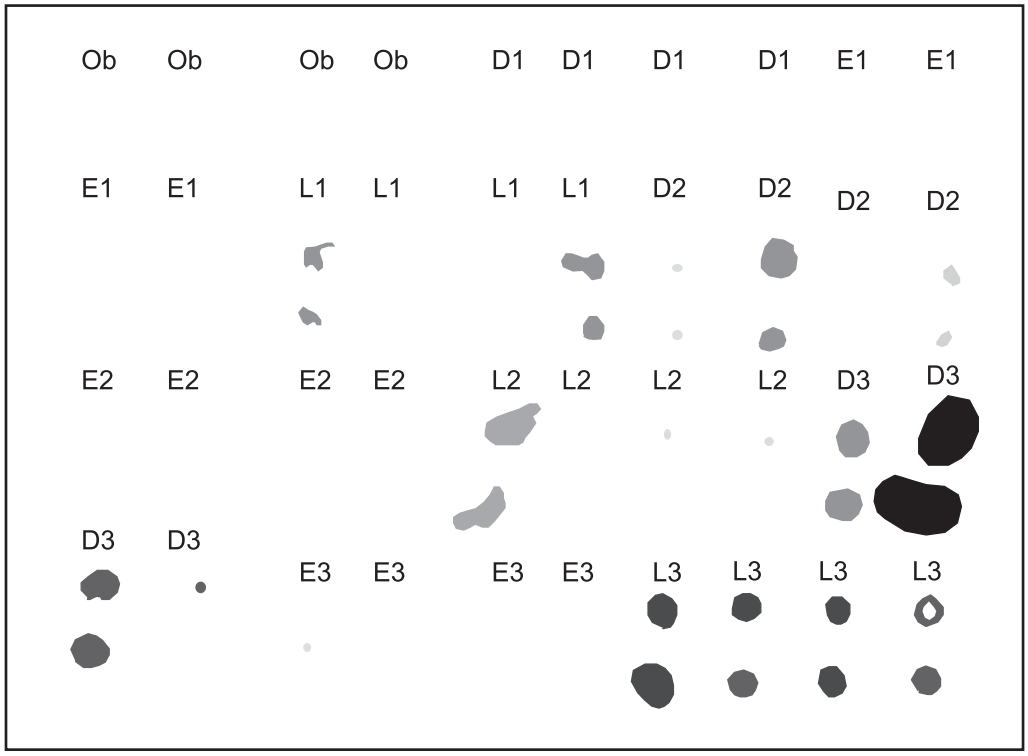

Fig. 1. – Dot blot experiment: photo of the dot blot membrane blotted with DNA from *Darwinula stevensoni*. The figure has been redrawn after the original photograph which is available online as supplementary material. Each dot represents one individual ostracod. The darkness of the dots corresponds to the amount of CPDs and thus the relative amount of DNA damage. Numbers correspond to hours of exposure: 1h (23kJ/m^2), 2h (47kJ/m^2) and 3h (70kJ/m^2). D = dead individuals; E = extracted DNA; L = living individuals; Ob = the positive controls.

example of the *hsp* gene). Data and figures for COI and ITS are not shown here because they are very similar to the results of *hsp* and also lack any clear patterns. They are available from the first author on request). Thus, the expected positive correlation of DNA damage to UVB dose is lacking. Furthermore, we observed high variability of relative DNA damage between the six replicas and lack of any difference between the nuclear and mitochondrial regions.

ELISA

In a preliminary ELISA pilot experiment, the three different types of material (alive, dead and DNA extractions), derived from ostracods collected in autumn, were each separately exposed to UVB. Under these conditions, DNA damage increased significantly with exposure duration (0kJ/m^2, 23kJ/m^2, 47kJ/m^2 and 70kJ/m^2) for the DNA extractions ($p=0.0016$, $H=14.47$, $df=15$) and the dead ostracods ($p=0.0012$, $H=18.07$, $df=15$). Post-hoc Tukey HSD tests revealed that UV damage in DNA extractions differed significantly between the three treatments groups ($p=0.0015$ to $p=0.0165$, $df=15$) while in dead ostracods, UV damage differed significantly between all four exposure times ($p=0.00015$ to $p=0.011$, $df=15$). We also found a positive correlation between the duration of exposure (UVB dose) and the relative amount of DNA damage in two of the three types of material ($R^2= 0.966$, $p<0.001$ for the DNA extractions and $R^2= 0.979$, $p<0.001$ for the dead ostracods). However, for the exposed living individuals, no significant difference in DNA damage was observed for different UVB doses and no significant correlation was found.

In a subsequent ELISA experiment (with animals sampled in spring), all three types of material were simultaneously exposed to UVB. This time, however, there were no significant differences or correlations between the different conditions (results not shown but available from the first author on request). Furthermore, the positive control (no UVB exposure) gave

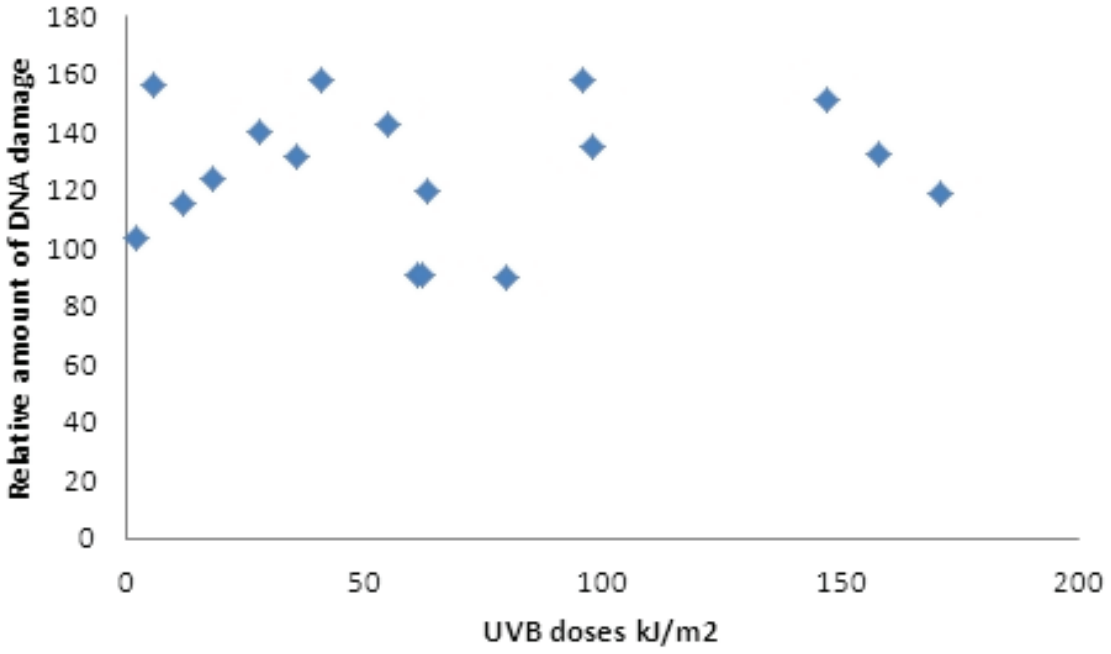

Fig. 2. – PI assay for part of the nuclear *hsp* gene of *Darwinula stevensoni*. The average, relative amount of DNA damage is plotted against the doses of UVB . The average, relative amount of DNA damage was calculated from the relative intensity of PCR products for all six replicas with the program Image J (Girish & Vijayalakshmi, 2004).

such high signals for UVB damage that it was no longer possible to subtract background levels. Also subsequent experiments with fresh products, new stocks of antibodies, and different DNA concentrations failed to show significant differences between the various treatments, and also did not give any clear correlation between the UVB doses and the amount of DNA damage.

Dot blot

As expected, this technique found no DNA damage in the positive controls not subjected to UVB exposure (Fig. 1). However, neither were CPDs detected from any DNA extractions, regardless of the UVB dose (Fig. 4). We also found no significant differences, with ONE-way ANOVAS, in the amount of DNA damage between dead or living ostracods having been exposed to three different UVB doses. With posthoc Tukey's HSD tests, however, a significant increase in DNA damage was found in both dead and living individuals depending on the duration of UVB exposure ($p=0.029$ and $p=0.003$ and $df=21$, respectively, for DNA damage in dead ostracods; $p=0.00016$ for both comparisons and $df=17$ for DNA damage in living ostracods, respectively). We also observed a positive correlation between DNA damage and UVB dose for the dead individuals, which was not statistically significant ($R^2= 0.960$, $p=0.09$). This first dot blot experiment was conducted with adult *D. stevensoni* collected in autumn. When we repeated the dot blot experiments with other ostracods in spring, we could not reproduce the results. Instead, we found high variability between replicas and failed to observe the expected positive correlation between DNA damage and UVB dose (results not shown but available from the first author on request).

Temperature-dependent experiment

After two hours of exposure at 4°C, all UVB-exposed ostracods were dead, while the unexposed ostracods were all still alive. Two hours of UVB exposure are equal to an UVB dose of 50kJ/m^2. Thus, the estimated relative lethal UVB dose for *D. stevensoni* at 4°C is at least 50kJ/m^2, while it was 130 kJ/m^2 at room temperature (Van den Broecke et al., 2012). This difference was statistically significant ($p<0.0001$; $df=9$, $Chi^2 = 429.31$).

DISCUSSION

With our PI assays, no clear correlation between UVB exposure and DNA damage was observed (Fig. 2), while Jenkins et al. (2000) did find such

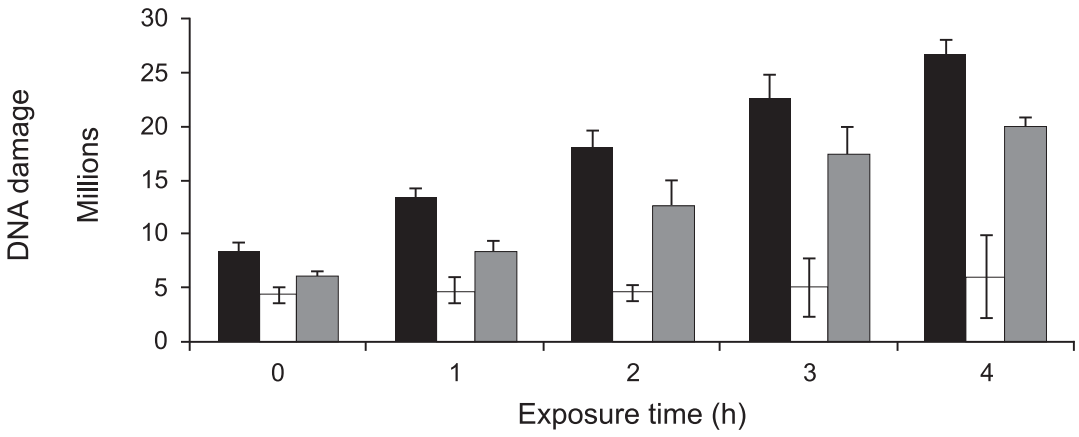

Fig. 3. – Pilot ELISA experiment for *Darwinula stevensoni* with DNA extractions (grey), dead (black) and living (white) ostracods being subsequently exposed to UVB. The numbers on the x axis are the hours of exposure [1h (23kJ/m^2), 2h (47kJ/m^2) and 3h (70kJ/m^2)], which are plotted against the average DNA damage and its standard deviation. Average DNA damage is calculated as the average number of CPDs from all replicas per treatment detected by VICTOR^{TM}.

a correlation when applying the same technique to mouse DNA. They investigated a larger PCR fragment of 1700bp although our three PCR regions together amount to 2100bp. The mouse DNA fragments were also much more sensitive to UVB damage as is illustrated by the maximal dose of 14kJ/m^2 that was used as compared to our maximal dose of 153kJ/m^2. Because only a limited region of the genome was analysed in our PI assays, chance might dictate whether the PCR target region of a few hundred basepairs is hit by UVB, and thus DNA damage is caused. This could also explain the large variability in relative DNA damage between the different replicas and genomic regions with the same UVB dose. It could also be that the response to UVB damage is too variable in the mixture of cells of which ostracods are composed as compared to the standardized animal cell cultures, which have been successfully used for PI assays (Govan et al., 1990; Kalinowski et al., 2000). Also, the PI-assay might be less suitable for investigating UV damage in living ostracods because of their high resistance to UVB. Ostracod valves block up to 80% of the UVB (Van den Broecke et al., 2012) and 60% in the case of *D. stevensoni*. It thus may be necessary to analyse larger parts of the genome to significantly increase the resolution power of the technique to quantify UVB damage.

Because of those concerns, we subsequently applied two other techniques, namely ELISA and dot blots, where DNA damage is detected in the entire genome. Because ostracods without valves die quickly, it was necessary to find other suitable material to test for the effect of ostracod valves in UVB protection (Van den Broecke et al., 2012). DNA extractions from *D. stevensoni*, which are not protected by valves, were exposed to UVB in the ELISA and dot blot experiments. We expected that UVB damage would be higher in DNA extractions than in living or dead ostracods. In the dot blot experiment, we found no CPDs in the extracted DNA and this could be due to the fact that the DNA was degraded. We also exposed dead individuals to UVB to test whether metabolic processes (Schön & Martens, 1998) might actively repair UVB damage in the DNA. In this case, we would expect more UVB damage in the dead ostracods than in the living ones. The preliminary results of the ELISA experiment, which we conducted in autumn, were promising. As expected, UVB damage increased with the UVB dose for all three conditions (Fig. 3). But when we repeated the experiment in spring exposing the three different kinds of material (DNA extractions, dead and living ostracods) simultaneously, the previous results could not be reproduced. Also the control without UVB exposure showed evidence for UVB damage and the previous, positive correlation between UVB dose and UVB damage was lacking. Furthermore, the various treatments showed high standard deviations. We tested for technical or contamination problems, but neither of these seems to be able to explain the inconsistency. Similarly for the dot blot experiments, the first results for the living and dead individuals followed our expectations (Fig. 4), but could not be repeated. This technique might have failed for the DNA extractions because DNA could have been degraded. For future experiments, it will be necessary to include positive controls to check for integrity of the DNA extractions used. As in the ELISA experiments, individuals for our different dot blot experiments were collected in different seasons (spring-summer). Studies on zooplankton have clearly demonstrated that the same species responds differently to UVB in different seasons (Strutzman, 1999; Tartarotti et al., 1999). Because of high intraspecific spatial and temporal variability, only results from the same lake and time should be compared when ranking species-specific UVB tolerances (Leech & Williamson, 2000). All individuals for our experiments came from the same water body but indeed, from different seasons. Because of the exceptionally long life cycle of *D. stevensoni* (1-4 years) and the low number of offspring per female (11-15 daughters) (Ranta, 1979; McGregor, 1969; Van Doninck et al., 2003), it has not yet been possible to establish laboratory cultures of *D. stevensoni* for UVB experiments. Such cultures may overcome possible seasonal and maternal

effects and should thus be established for similar experiments in the future.

An additional complicating factor in the investigation of UVB damage may be age differences of the exposed ostracods. Various studies have shown that adults of zooplankton tolerate UVB better than juvenile stages (Leeck & Williamson, 2000; Vega & Pizarro, 2000; Ramos-Jiliberto et al., 2004; Huebner et al., 2006). One of the few documented exceptions to this pattern is the higher adult mortality in the rotifer *Asplanchna girodi* compared with its juveniles (Grad et al., 2003). The life cycle of *Darwinula stevensoni* in Belgium as about one year (Van Doninck et al., 2003) and thus also in Hollandersgatkreek from where our material was collected, and up to 4 years in subarctic areas (Ranta, 1979). We used only one life stage, namely adults without embryos in our experiments, but since the individuals came from a natural population, we have no information on the actual age of the exposed ostracods. Van Doninck et al. (2003) and Ranta (1979) found that old and young adults coexisted during spring, which may have further contributed to the lack of reproducibility between our various ELISA and dot blot experiments. It is unfortunately not possible to derive the age of adult *D. stevensoni* from their body size or valve outlines. Therefore, it would probably be best to conduct future experiments in autumn, when only one age class of adult *D. stevensoni* is present. Furthermore, during such controlled sampling, the temperature and ambient UVB of the water body from which the ostracods are taken should also be measured.

The estimated relative lethal UVB dose for *D. stevensoni* was significantly larger at room temperature (130kJ/m^2; Van den Broecke et al., 2012) than at 4°C (50kJ/m$^{2)}$. In studies on a crab, *Cyrtograpsus* sp. (Moresino & Helbling, 2010), on *Daphnia catawba* and *Leptodiaptomus minutus* (Williamson et al., 2002), on *Daphnia pulicaria* (MacFadyen, 2004), and on *Evechinus chloroticus* and *Diadema setosum* (Lamare et al., 2006), mortality after UVB exposure was significantly higher at 15°C than at 20°C. These results all suggest that UVB tolerance is temperature-dependent because it involves enzymatic repair of UVB damage. Our result reflects either adaptation to repair at higher temperatures in *Darwinula stevensoni* and/or the presence of some kind of metabolic, enzymatic process providing protection against UVB damage. In the latter case this process would be active to a larger extent at room temperature than at 4°C as indicated by the significantly lower estimated relative lethal UVB dose at 4°C. Enzymatic repair of UVB damage is thus expected to be faster and more efficient at 20°C, which may explain why the estimated relative

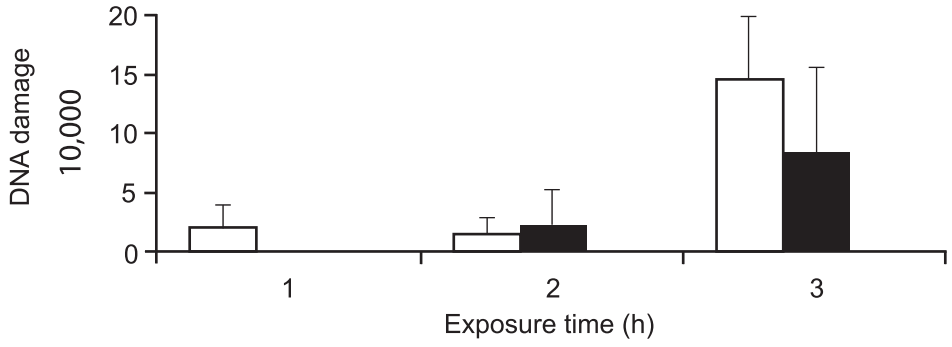

Fig. 4. – Pilot dot blot experiment for *Darwinula stevensoni* with DNA extractions (grey), dead (black) and living (white) ostracods. The numbers on the x-axis are hours of exposure [1h (23kJ/m^2), 2h (47kJ/m^2) and 3h (70kJ/m^2)], which are plotted against the average DNA damage and its standard deviation. Average DNA damage was calculated from the relative intensity of dots on the membrane from all replicas per treatment with the program Image J (Girish & Vijayalakshmi, 2004). Please note that we did not detect any DNA damage in the DNA extractions in the pilot dot blot experiment.

lethal doses of *D. stevensoni* at room temperature are similar to the estimated relative lethal doses of other non-marine ostracods despite the valves of *D. stevensoni* providing less protection (Van den Broecke et al., 2012). Our estimated relative lethal dose at 4°C could resemble the actual lethal dose of UVB for *D. stevensoni* without DNA repair or at suboptimal temperature conditions. Highly efficient and fast DNA repair during our exposure experiments is another plausible explanation for the variability of our results on the living individuals of *D. stevensoni* and for the absence of a positive correlation between DNA damage and duration of UVB exposure in living ostracods. In invertebrates, two important repair processes are known: nucleotide excision repair (NER, dark repair) and photo-enzymatic repair (PER, light repair) (Sancar, 1994a). NER is an energetically costly complex multi-protein, multi-step pathway, and is found in almost all taxa without being specific to UVB-induced DNA damage (Sinha & Häder, 2002). PER uses the enzyme photolyase and can reverse pyrimidine dimers (Sutherland, 1981; Mitchell & Karentz, 1993). Since it is a single-enzymatic process driven by photorepair radiation, it is less costly than NER (MacFadyen et al., 2004). Although PER is specific to UV-induced DNA damage, it is not present in all eukaryotic taxa investigated so far (Sancar, 1994b). PER and NER are also temperature-dependent mechanisms with more repair at higher temperatures (Williamson et al., 2002). Survival of UV-stressed *Daphnia* increased in the presence of PER (Siebeck & Böhm, 1991; Grad et al., 2001; Williamson et al., 2001, 2002; Huebner et al., 2006). A study by MacFadyen et al. (2004) provided additional evidence for PER in *Daphnia* at the molecular level. Other zooplankton taxa such as the rotifer *Asplanchna girodi* seem to utilise NER and have little to no PER (Sawada & Enesco, 1984; Grad et al., 2001). In juvenile *A. girodi*, however, evidence for PER has been found (Williamson et al., 2002; Grad et al., 2003). The importance of repair processes in copepods is not well understood and PER seems to be patchily distributed: both the cyclopoid copepod *Metacyclops mendocinus* and the calanoid copepod *Leptodiaptomus minutus* showed evidence for PER (Gonçalves et al., 2002; Williamson et al., 2002). Also Zagarese et al. (1997) found that PER accounted for the relatively high UVB tolerance in red *Boeckella gibbosa*, while little evidence of PER was found in *Boeckella gracilipes* (Zagarese et al., 1997; Tartarotti et al., 2000). Surprisingly, when exposing four different species of *Daphnia* to a single acute UVB dose, higher survival and repair rates were found at the lower experimental temperature (10°C compared to 20°C), indicating that the enhanced rate of PER at lower temperature contributed significantly to the recovery of these animals (Connelly et al., 2009). The same authors also confirmed that photorepair was the primary mechanism to remove DNA lesions in *Daphnia*. For *Darwinula stevensoni*, further research, for example using qPCRs, is necessary to identify which system of repair from UVB damage is active. Controls kept in the dark (thus preventing PER DNA repair) will be needed to test whether the lack of DNA damage is due to effective sunscreening in photorepair or to effective PER repair.

CONCLUSIONS

To conclude, there are many factors that must be considered when investigating the response to UVB in ostracods or other invertebrates. The valves are important in the protection against UVB (Van den Broecke et al., 2012) but also other factors such as seasonality (Strutzman, 1999; Tartarotti et al., 1999), age (e.g. Huebner et al., 2006) and temperature (Moresino & Helbling, 2010; Rautio & Tartarotti, 2010) of the habitat and during the experiment, are obviously of great importance and should be carefully controlled in future experimental set-ups for investigating UVB damage in living or dead ostracods. Future experiments could be further facilitated by using animals from lab cultures, which would overcome any possible maternal effects. Finally, repeating the experiments under a range of

different temperature conditions would help to determine whether the higher UVB damage in *D. stevensoni* at 4°C reflects a general adaptation of the species to higher temperatures or indicates the presence of temperature-dependent, metabolic repair processes.

ACKNOWLEDGEMENTS

We thank Claudine Devries-Duchene and Zohra Elouaazizi for technical support. Prof Dirk Verschuren (Ugent) is acknowledged for the use of his cold chamber for the temperature-dependent experiment. This research was funded by the Belgian Federal Science Policy (Belspo WI/36/I03 and MO/36/015).

REFERENCES

Butlin RK, Schön I & Griffiths HI (1998). Introduction to reproductive modes. *In*: Martens K (ed), Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods. Backhuys Publishers, Leiden:1-24.

Connelly SJ, Moeller RE, Sanchez G & Mitchell DL (2009). Temperature effects on survival and DNA repair in four freshwater cladoceran *Daphnia* species exposed to UV radiation. Photochemistry and Photobiology, 85: 144-152.

Friedberg EC, Walker GC & Siede W (1995). DNA Repair and Mutagenesis. ASM Press, Washington DC.

Geiger W (1998). Population dynamics, life histories and reproductive modes. *In*: Martens K (ed), Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods. Backhuys Publishers, Leiden:215-228.

Girish V & Vijayalakshmi A (2004). Affordable image analysis using NIH Image/Image J. Indian Journal of Cancer, 41:47.

Gonçalves RJ, Villafañe VE & Helbling EW (2002). Photorepair activity and protective compounds in two freshwater zooplankton species (*Daphnia menucoensis* and *Metacyclops mendocInus*) from Patagonia, Argentina. Photochemical & Photobiological Sciences, 1:996-1000.

Govan HL, Valles-Ayoub Y & Braun J (1990). Fine-mapping of DNA damage and repair in specific genomic segments. Nucleic Acids Research, 18:3823-3830.

Grad G, Burnett BJ & Williamson CE (2003). UV damage and photoreactivation: timing and age are everything. Photochemistry and Photobiology, 78:225-227.

Grad, G, Williamson CE & Karapelou DM (2001). Zooplankton survival and reproduction responses to damaging UV radiation: a test of reciprocity and photoenzymatic repair. Limnology and Oceanography, 46:584-591.

Hessen DO (1996). Competitive trade-off strategies in Arctic *Daphnia* linked to melanism and UV-B stress. Polar Biology, 16:573-576.

Holmes J.A & Chivas A (2002). The Ostracoda: Applications in Quaternary Research. American Geophysical Union, Washington DC.

Horne d, Homes j, Finnberg v & Rodriquez-Lazaro j (2012) Ostracoda as Proxies for Quaternary Climate Change, Volume 17 (Developments in Quaternary Science).

Huebner JD, Young DLW, Loadman NL, Lentz VJ & Wiegand MD (2006). Age-dependent survival, reproduction and photorepair activity in *Daphnia magna* (Straus, 1820) after exposure to artificial ultraviolet radiation. Photochemistry and Photobiology, 82:1656-1661.

Hurtubise RD, Havel JE & Little EE (1998). The effects of ultraviolet-B radiation on freshwater invertebrates: experiments with a solar simulator. Limnology and Oceanography, 43:1082-1088.

Jagger J & Stafford RS (1965). Evidence for Two Mechanisms of Photoreactivation in *Escherichia coli* B. Biophysical Journal, 5:75-88.

Jenkins GJS, Burlinson B & Parry JM (2000). The Polymerase Inhibition Assay: a Methodology for the Identification of DNA-damage Agents. Molecular Carcinogenesis, 27:289-297.

Kalinowski DP, McCready SJ, Osman F & Yasui A (2000). Repair of UV damage in the fission yeast *Schizosaccharomyces pombe*. Mutation Research, 251:197-210.

Lamare MD, Barker MF, Lesser MP & Marshall C (2006). DNA photorepair in echinoid embryos: Effects of temperature on repair rate in Antarctic and non-Antarctic species. Journal of Experimental Biology, 209:5017-5028.

Leech DM & Williamson CE (2000). Is tolerance to ultraviolet radiation in zooplankton related to body size, taxon, or lake transparency? Journal of Applied Ecology, 10:1530-1540.

MacFadyen EJ, Williamson CE, Grad G, Lowery M, Jeffrey WH & Mitchell DL (2004). Molecular response to climate change: temperature dependence of UV- induced DNA damage and repair in the freshwater crustacean *Daphnia pulicaria*. Global Change Biology, 10: 408-416.

Martens K (1998). Sex and Parthenogenesis – Evolutionary Ecology of Reproductive Modes in Non-marine Ostracods. Backhuys Publishers, Leiden.

Martens K, Rossetti G & Horne DJ (2003). How ancient are ancient asexuals? Proceedings of the Royal Society B: Biological Sciences, 270:723-729.

Martens K, Schön I, Meisch C & Horne DJ (2008). Global diversity of ostracods (Ostracoda, Crustacea) in freshwater. Hydrobiologia, 595: 185-193.

McGRegor DL (1969). The reproductive potential, life history and parasitism of the freshwater ostracod *DarwInula stevensoni*. *In*: Neale JW (ed), The taxonomy, morphology and ecology of recent Ostracoda. Oliver & Boyd, Edinburgh: 194-221.

Mitchell DL & Karentz D (1993). The induction and repair of DNA photodamage in the environment. *In*: Young AR, Björn LO, Moan J & Nultsch W (eds), Environmental UV photobiology, Plenum Press:345-371.

Moresino RDH & Helbling EW (2010). Combined effects of UVR and temperature on the survival of crab larvae (Zoea I) from Patagonia: The role of UV-absorbing compounds. Marine Drugs, 8(5): 1681-1698.

Ramos-Jiliberto R, Dauelsberg P & Zúñiga LR (2004). Differential tolerance to ultraviolet-B light and photoenzymatic repair in cladocerans in a Chilean lake. Marine & Freshwater Research, 55:193-200.

Ranta E (1979). Population biology of *DarwInula stevensoni* (Crustacea, Ostracoda) in an oligotrophic lake. Annales Zoologici Fennici, 16:28-35.

Rautio M & Tartarotti B (2010). UV radiation and freshwater zooplankton: damage, protection and recovery. Freshwater Reviews, 3(2):105-131.

Sancar A (1994a). Mechanisms of DNA excision repair. Science, 266:1954-1956.

Sancar A (1994b). Structure and function of DNA photolyase. Biochemistry, 33:2-9.

Sancar A & Tang M (1993). Nucleotide excision repair. Photochemistry and Photobiology, 57: 905-921.

Sawada M & Enesco HE (1984). Effects of UV radiation on the lifespan of the rotifer *Asplanchna brightwelli*. Experimental Gerontology, 19:289-296.

Schön I & Martens K (1998). DNA repair in ancient asexuals – a new solution to an old problem? Journal of Natural History, 32:943-948.

Schön I & Martens K (2003). No slave to sex. Proceedings of the Royal Society B: Biological Sciences, 270:827-833.

Schön I, Rossetti G & Martens K (2009). Darwinulid ostracods: ancient scandals or scandalous gossip. *In*: Schön I, Martens K & van Dijk P (eds), Lost sex. Springer, The Netherlands:217-240.

Schön I, Butlin RK, Griffiths HI & Martens K (1998). Slow molecular evolution in an ancient asexual ostracod. Proceedings of the Royal Society B: Biological Sciences, 265:235-242.

Setlow RB, Grist E, Thompson K & Woodhead AD (1993). Wavelengths effective in induction of malignant melanoma. Proceedings of the National Academy of Sciences of the United States of America, 90:6666-6670.

Siebeck O & Böhm U (1991). UV-B effects on aquatic animals. Verhandlungen der Internationalen Vereinigung für theoretische und angewandte Limnologie, 24:2773-2777.

Sinha RP & Häder D-P (2002). UV-induced DNA damage and repair: a review. Photochemical & Photobiological Sciences, 1:225-236.

Sinha RP, Dautz M & Häder D-P (2001). A simple and efficient method for the quantitative analysis of thymine dimers in Cyanobacteria, Phytoplankton and Macroalgae. Acta Protozoologica, 40:187-195.

Straub EB (1952). Mikropaläontologische Untersuchungen im Tertiär zwischen Ehingen und Ulm a.d. Donau. Geologisches Jahrbuch, 66:433-523.

Strutzman PL (1999). A comparative study of ultraviolet radiation in different populations of *Diaptomus mInutes*. Journal of Plankton Research, 21:387-400.

SUTHERLAND BM (1981). Photoreactivation. Bioscience, 31:439-444.

TARTAROTTI B, CABRERA S, PSENNER R & SOMMARUGA R (1999). Survivorship of *Cyclops abyssorum tatricus* (Cyclopoida, Copepoda) and *Boeckella gracilipes* (Calanoida, Copepoda) under ambient levels of solar UVB radiation in two high-mountain lakes. Journal of Plankton Research, 21:549-560.

TARTAROTTI B, CRAVERO W & ZAGARESE HE (2000). Biological weighting function for the mortality of *Boeckella gracilipes* (Copepods, Crustacea) derived from experiments with natural solar radiation. Photochemistry and Photobiology, 73:314-319.

VAN DEN BROECKE L, MARTENS K, PIERI V & SCHÖN I (2012). Ostracod valves as efficient UV protection. Journal of Limnology, 71(1):119-124.

VAN DONINCK K, SCHÖN I, DE BRUYN L & MARTENS K (2002). A general purpose genotype in an ancient asexual. Oecologia, 132:205-212.

VAN DONINCK K, SCHÖN I, MARTENS K & GODDEERIS B (2003). The life-cycle of the ancient asexual ostracod *DarwInula stevensoni* (Brady & Robertson, 1870) (Crustacea, Ostracoda) in a temporate pond. Hydrobiologia, 500:331-340.

VEGA MP & PIZARRO R (2000). Lethal effect induced by ultraviolet-B in a planktonic copepod: role of the post-irradiation time on mortality measurements. Journal of Freshwater Ecology, 15:1-5.

WILLIAMSON CE, GRAD G, DE LANGE HJ, GILROY S & KARAPELOU DM (2002). Temperature-dependent ultraviolet responses in zooplankton: implications of climate change. Limnology and Oceanography, 47:1844-1848.

WILLIAMSON CE, NEALE PJ, GRAD G, DE LANGE HJ & HARGREAVES BR (2001). Beneficial and detrimental effects of UV on aquatic organisms: implications of spectral variation. Journal of Applied Ecology, 11:1843-1857.

ZAGARESE HE, FELDMAN M & WILLIAMSON CE (1997). UV-B induced damage and photoreactivation in three species of *Boeckella* (Copepods, Calanoida). Journal of Plankton Research, 19:357-367.

Received: May 15th, 2012

Accepted: July 19th, 2013

Branch editor: Adriaens Dominique