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Melanin and antipredatory defenses in *Daphnia dadayana* under UVR exposure

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Abstract

Ultraviolet radiation (UVR) exposure has potentially hazardous effects on aquatic life, even more in the southern hemisphere, which is close to ozone layer depletion. Aquatic animals living in shallow water cannot escape from UVR effects swimming down, so they have to generate other traits to confront it (i.e., enzymes or pigments). *Daphnia* is a worldwide freshwater genus that inhabits ponds and lakes. *Daphnia dadayana* inhabits shallow lakes in Patagonia presenting a yellowish carapace and a horn-like structure in juveniles assumed for avoiding invertebrate predator attacks. We aimed to determine the effect of UVR exposure on the accumulation of melanin and if the development of the antipredatory defense affects the antioxidant response (glutathione S-transferase [GST] activity) to UVR. We carried out laboratory experiments with treatments with and without UVR exposure measuring melanin accumulation by photographic analyses. Also, we performed an experiment to generate the antipredatory structure exposing *D. dadayana* indirectly to the predaceous copepod *Parabroteas sarsi*. Our results showed that UVR increased melanin accumulation in *D. dadayana* and that the morphological structure against predators did not decrease the antioxidant enzymatic defenses (GST). Our concluding remarks are that *D. dadayana* is a successful organism that can use its phenotypic plasticity to cope with environmental stressors such as invertebrate predators and UVR exposure with no trade-off between these two stressors.

KEYWORDS

biomarkers, phenotypic plasticity, pigments, ultraviolet radiation, zooplankton

1 | INTRODUCTION

Studies on interacting variables represent more accurately environmental conditions than only a one-factor experiment (Wernberg, Smale, & Thomsen, 2012). Predator presence and ultraviolet radiation (UVR) are critical ecological factors affecting zooplankton populations. In response to these environmental variables, zooplankton develop physiological, morphological, and behavioral strategies (Gabriel, 2005; Tollrian & Heibl, 2004), including the capacity to generate antipredation structures to avoid invertebrate predation (Dodson, 1974, 1984; Jeschke, Kopp, & Tollrian, 2002; Pijanowska, 1992) and the accumulation of photoprotective pigments to deal with UVR effects (Borgeraas & Hessen, 2002; Hansson & Hylander, 2009; Tollrian & Heibl, 2004).

Exposure to UVR potentially damages zooplankton cellular structures like DNA, proteins, and lipids. These short wavelengths are effectively harmful, and normal levels of UVR in temperate areas are associated with increased mortality in zooplankton

(Hernández Moresino & Helbling, 2010; Leech & Williamson, 2000; Zagarese & Williamson, 1994). UVR is a critical variable for zooplankton from shallow lakes because they cannot perform vertical migrations to avoid these harmful short wavelengths, as does the zooplankton from deep lakes (Williamson, Fischer, Bollens, Overholt, & Breckenridge, 2011). To deal with UVR, zooplankton from shallow lakes with no fish can either synthesize melanin to prevent UVR damage (Cladocera; Herbert & Emery, 1990) or accumulate photoprotective compounds from their food (mostly algae) and store them in the animal body as micosporine like amino acids (MAAs) and carotenoids (Copepods; Hairston, 1976; Hylander, Kiørboe, Snoeijs, Sommaruga, & Nielsen, 2015; Moeller, Gilroy, Williamson, Grad, & Sommaruga, 2005). *Daphnia* melanin synthesis and concentration is directly related to the UVR dose that animals receive (Hansson, Hylander, & Sommaruga, 2007) and melanin concentration declines in animals raised without the UVR light portion. Melanin pigmentation is heterogeneous in the animal body surface, concentrating its maximum in the dorsal neck area of the carapace that protects the vulnerable digestive tube when swimming in dorsal position (Herbert & Emery, 1990; Hessen, Borgeraas, Kessler, & Refseth, 1999; Luecke & O'Brien, 1983).

Even though shallow lakes are usually "protected" from UVR direct effects on biota due to their high dissolved organic carbon (DOC) content, short-wavelength absorption by DOC generates reactive oxygen species (ROS) that are highly reactive and potentially damaging for the biota (Souza, Modenutti, & Balseiro, 2007; Wolf, Andersen, Hessen, & Hylland, 2017). UVR exposure increases the antioxidant response where enzymatic antioxidants like catalase neutralize ROS generated by UVR photons that interact with organic matter and H_2O (Borgeraas & Hessen, 2002; Häder, Helbling, Williamson, & Worrest, 2011). Other antioxidants like glutathione S-transferase (GST) act as detoxifiers of the byproducts created by ROS; thus, GST activity increases if there is a damage risk in the cell by the increase of ROS (Souza et al., 2007). Moreover, pigmentation and antioxidant enzymes in copepods are flexible defense systems only induced when needed (Hylander, Souza, Balseiro, Modenutti, & Hansson, 2012).

Phenotypic plasticity is the ability to express different phenotypes induced as a response to an environmental signal, mostly chemical signals, and must be received in a specific developmental moment, to allow the organism to generate the appropriate response (Agrawal, 2001). This phenotypic expression implies a trade-off between the investment of energy on growth or developing a trait to cope with a specific environmental stressor. Antipredator traits represent an excellent example of phenotypic plasticity. *Daphnia* species can develop different morphological defense structures, such as neck-teeth and crests, in the presence of invertebrate predators such as *Chaoborus* or *Notonecta* (Dodson & Havel, 1988; Hebert & Grewe, 1985; O'Brien, 1979). Other defensive structures (i.e., spines) increase the apparent size to hinder prey manipulation by the predator (Balseiro & Vega, 1994; Dodson, 1988; Dodson & Havel, 1988; Hanazato, 1990; Hebert & Grewe, 1985). Inducible defenses formation in *Daphnia* occur by the reception of chemical compounds, called *kairomones*, that are released by the predator (Tollrian & Dodson, 1999). However, these signals must be received during the late-embryonic stage of the parthenogenetic egg development when neonates are still inside the mother's carapace (Imai, Naraki, Tochinai, & Miura, 2009). At that moment, a chain of biological reactions generates neural signals that activate endocrinal systems which, in turn, induce changes in the expression of morphogenetic factors resulting in the formation of these defense structures in neonates (Miyakawa et al., 2010). However, there is a physiological cost associated with the production of these structures with a demographic decay (Black & Dodson, 1990; Riessen & Sprules, 1990). Thus, the physiological cost of the generation of antipredator defenses could affect other defensive mechanisms against other stressors, like the antioxidant response under UVR exposure.

The cladoceran, *Daphnia dadayana* inhabits shallow lakes and was described with a hyaline to yellowish carapace and juveniles with a horn-like head in the first two instars (Paggi, 1999). This cladoceran coexists with *Parabroteas sarsi*, a big predaceous calanoid copepod (5 mm) that can access a great variety of prey including the genus *Daphnia* (Vega, 1995). Early studies have indicated that *P. sarsi* induces allometric growth of the caudal spine in *Daphnia* spp. (Balseiro & Vega, 1994; Vega, 1995). We recently detected a brown-colored population of *D. dadayana* in the small shallow lake Los Juncos (Patagonia) coexisting with *P. sarsi*. Considering that melanin synthesis and antipredator defense structures are plastic, our main aim here was to determine if these two defenses interact with an antioxidant enzyme under UVR exposure. We predict that melanin will act as the first barrier such as a UVR shield; therefore, GST activity will not be increased in pigmented individuals when exposed to UVR. In addition, we also predict that the investment in an antipredatory structure will negatively affect the antioxidant response capacity (GST activity).

2 | METHODS

D. dadayana was sampled from Los Juncos Lake (41° 04´S 71°W), a temporal water body, located in the Patagonian plateau at 911 m above sea level. The surrounding area is a steppe that is used for sheep breeding. Precipitations (around 550 mm a year, mainly in autumn and winter) constitute the main water input to the environment. The lake has a hydroperiod from June to January that can be modified by human activities (water diversion for sheep breeding). The lake surface during the hydroperiod is around 2 ha with a maximum depth of 1 m. The water is slighty alkaline (pH 7.8) and the conductivity is 500 μS/m. The lake is fishless and the zooplankton is constituted by rotifers, copepods (i.e., *P. sarsi* and other Boeckellids), cladocerans (*D. dadayana*), and some insect species (*Notonecta* sp.; Modenutti, Balseiro, Dieguez, Queimalinos, & Albarino, 1998).

D. dadayana individuals were raised under laboratory-controlled conditions of light, temperature, and food (see below). We designed two different types of experiments to analyze (a) the melanin production under UVR exposure and the effect on antioxidant defense

(GST activity) and (b) if the investment in antipredatory protection reduces the antioxidant defense response (GST activity) under UVR exposure.

2.1 | Melanin production under UVR exposure

Mothers from the same cohort of *D. dadayana* were maintained in COMBO culture medium (Kilham, Kreeger, Lynn, Goulden, & Herrera, 1998) under controlled laboratory conditions (15°C of temperature, a photoperiod of 12:12 hr light:dark) and fed reactive oxygen species with *Chlamydomonas reindhartii* culture (MBL) to allow them to develop eggs over at least five generations before starting experimentation. For experimentation, we selected neonates born in a 12-hr period, and they were carefully placed with a pippete in a new medium with food (algae: *C. reindhartii*) concentration of 1.5 mg of carbon (C)/L. Immediately, the neonates were randomly disposed into two treatments: UVR and PAR (photosynthetic active radiation) exposure. For this purpose, we used 150 ml quartz flasks with 10 neonates each and exposed them to a 12:12 hr photoperiod with PAR and 2 hr of UVR (centered in the PAR period) every day, over 6 days (UVR treatment). That is, the treatment had 5 hr of PAR, 2 hr of PAR + UVR, 5 hr of PAR, and 12 hr darkness. The corresponding control (PAR treatment) was equally prepared in glass flasks and covered with cellulose acetate to avoid any UVR effect (see Section 2.4 below). There were four replicates of each treatment. In the first, third, and sixth days of the experimentation, we analyzed melanization from the lateral photographs taken from each animal and treatments (UVR exposure vs. PAR exposure). Photographs were obtained with a stereoscopic microscope (Olympus SZX9) attached to a digital photographic camera (5Mp). The TIFF image format was used to determine color intensity in the dorsal neck area (Figure 1) with ImageJ/FIJI software (Schindelin et al., 2012) in red, green and blue (RGB) color composition. These images were normalized to be comparable with the BaSic application developed for ImageJ by Peng et al. (2017). We expressed the color intensity in numerical values for colored RGB formats transformed into black and white. Black color corresponds to 0 and white to 255; therefore, animals with lower numbers show a higher intensity of melanization. Finally, on Day 6, we placed all the animals in Eppendorf tubes that were kept in a −80°C ultra freezer for further GST activity determinations.

2.2 | Antipredator defenses under UVR

To test differences between individuals with and without antipredatory defenses under UVR exposure, we performed another experimental set. We carried out two combined experiments, one for inducing the horn formation and a subsequent one with UVR exposure. To obtain neonates with antipredatory defenses (horn), we used the predaceous calanoid copepod *P. sarsi* as a source of *kairomones*. We sampled *P. sarsi* from Lake Los Juncos 1 day before the experiment started and fed them with *D. dadayana* conspecific to enhance the alarm signal in the medium, following Laforsch, Beccara, and Tollrian (2006).

To start the induction experiment, *Daphnia* mothers from the same cohort fed ad libitum under two treatments: (a) with predator signal and (b) without signal. We placed 15 *Daphnia* mothers in 2 L flasks in three replicates each for the two treatments. To each flask of the treatment with a predator signal, we added 10 *P. sarsi* in a tube isolated with a 200 μm mesh. The mesh was necessary to avoid direct predation but allowing water exchange (*kairomones*).

Once the neonates were born, we confirmed the presence of the helmet in the *kairomones* treatment, while we did not see an inducible defense in the treatment without predator. Twenty-four neonates of each treatment were transferred to the experimental flask for 3 days to allow growth, with 1.5 mg of C per L of food (*C. reindhartii*). On the third day, the induced neonates (born with helmet) were split into two groups, each one consisting of four replicates of three individuals. One group (four replicates), placed in quartz flasks, was exposed to UVR for 2 hr. The other group was transferred to glass flasks and covered with cellulose acetate to avoid UVR exposure. We applied the same procedure for the noninduced neonates (without defense; Figure 2). After 2 hr of UVR exposure we placed all individuals in Eppendorf tubes and stored at −80°C for further enzymatic and size analyses.

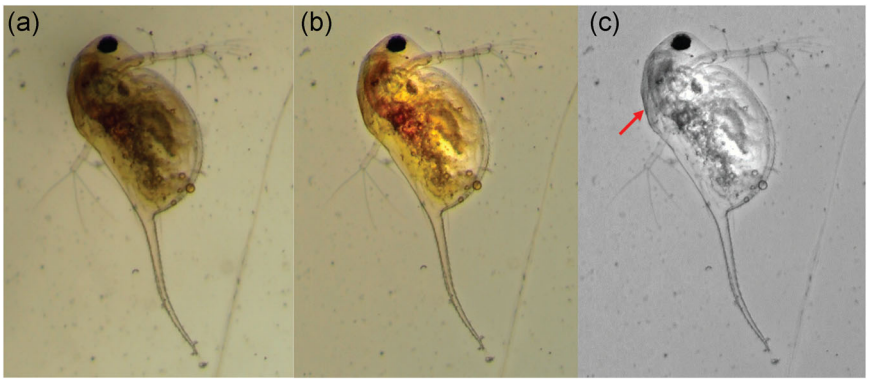

FIGURE 1 *Daphnia dadayana* photographs in (a) original RGB format, (b) normalized, and (c) transformed to black and white to measure. The red arrow in photograph (c) indicates the measuring point used in every sample for color intensity

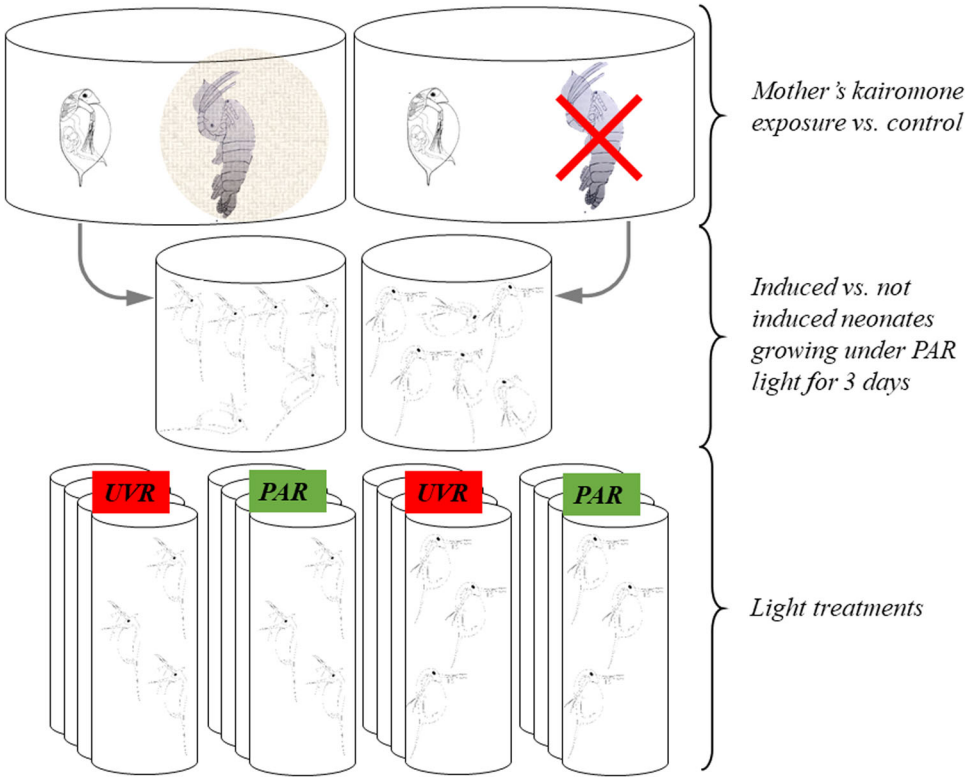

FIGURE 2 Schematic experimental diagram of the antipredatory defenses experiment and the subsequent UVR exposure experiment. PAR, photosynthetic active radiation; UVR, ultraviolet radiation

2.3 | Morphological determinations

We took lateral photographs of *D. dadayana* resulting from the experiment. This procedure was performed with different neonates from the four treatments to compare the size and shape (Figure 3) between animals exposed or not to the predator signal. We analyzed the photographs with Image-Pro Plus software (Media Cybernetics).

2.4 | Light features

UVR was provided by a light source consisting of two UVA340 fluorescent lamps (Q-Panel Lab Products), two daylight fluorescent lamps (Philips TLT 40 W), and two black light fluorescent lamps (UVA340 lamp from Q-Panel Lab Products, with maximum emission at 380 nm). The UV spectrum of the UVA340 light closely resembles the solar range between 280 and 350 nm (Shick, Romaine-Lioud, Ferrier-Pagés, & Gattuso, 1999). The black light was included to fill the gap between the maximum emission of UVA (340 nm) and the daylight fluorescent lamps (400 nm). During the incubation, the experimental set surface received 35 $\mu W \cdot cm^2 \cdot nm^{-1}$ of 340 nm wave-band, an irradiance level of the waveband that is equivalent to the surface sunlight in Andean lakes during summer (Modenutti, Balseiro, Callieri, Bertoni, & Queimaliños, 2005). The total 340-nm wave-band dose per day was of 2,520 J m^2.

2.5 | Biochemical determinations

As an antioxidant defense, we determined the GST activity. Each individual sampled was homogenized with an Ultrasonic Homogenizer (with one cycle, 40% of amplitude, and 2 mm Ø point; Sartorius, LABSONIC M) in 50 µl buffer solution (Tris-Sacarose, pH 7.4) and then incubated on ice for 10 min with 50 µl lysis buffer. Afterward, samples were centrifuged at 13,000*g* for 5 min at 4°C to obtain the supernatant (enzyme source). GST activity was determined adding to the sample (50 µl) 4 µl of GSH and 0.5 µl of staurosporine as substrate (fluorometric glutathione assay kit

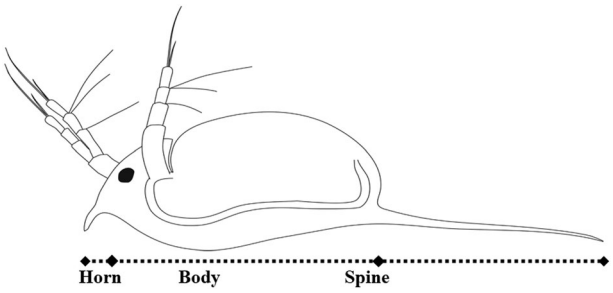

FIGURE 3 Measurements diagram of *Daphnia dadayana* (with antipredator defense in this figure) used to determine the criteria for the applied methodology. Horn plus spine corresponds to the whole defense

(CS1020); Sigma-Aldrich, St. Louis, MO, following manufacturer instructions). Then, the GST activity was estimated by fluorescence, read in a Perkin-Elmer LS45, excitation at 390 nm (10 nm bandwidth) and emission at 478 nm (10 nm bandwidth) each second for 2 min. We expressed the GST activity as micromoles of product per minute per individual.

2.6 | Statistical analysis

2.6.1 | For "melanin production under UVR exposure"

Melanin (color intensity) differences in *D. dadayana* between treatment and control in Days 1, 3, and 6 were analyzed with an ANCOVA (factor: light and co-variable: time), followed by the multiple comparisons Tukey a posteriori method ($\alpha = .05$). This statistical analysis was performed in R Studio (R Core Team, 2012). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, http://www.rstudio.com/. To compare GST activity in *D. dadayana* after UVR exposure in the last day of experimentation, we performed a *t* test between treatment and control, using Sigma Plot 12.5 (Systat Software Inc., San Jose, CA, EE.UU.).

To compare somatic size differences (total length, body length, and defense length) between induced or not neonates, we applied a *t* test. Finally, to compare GST activity of *D. dadayana* induced or not under UVR versus PAR treatment, we used a two-way ANOVA (factor A: induced defense and factor B: light). We performed all analyses in Sigma Plot 12.5 (Systat Software Inc., San Jose, CA).

3 | RESULTS

3.1 | Melanin production under UVR exposure

The photographic analysis allowed us to determine the melanization of the *D. dadayana* carapace under UVR exposure (Figure 4). On the first day, before exposure to UVR, there were no differences between both treatments (UVR vs. PAR). However, after the third day of UVR exposure, we observed a significant decrease in the brightness intensity (ANCOVA $F_{2,24} = 16.69$, $p < .001$; Figure 5) and this difference was enhanced on the sixth day (a posteriori Tukey light × Day 1, $p = .841$; light × Day 3, $p = .001$; light × Day 6, $p < .001$; Figure 5). On the other hand, there were no differences in animals in the PAR treatment over the 6 days of experimentation, maintaining the same brightness intensity since the first measurement.

On the sixth day of experimentation, we determined the GST activity for both, UVR and PAR treatments, and there were no significant differences between treatments (*t* test; $t = 1.439$, GL = 14, $p = .172$; Figure 6).

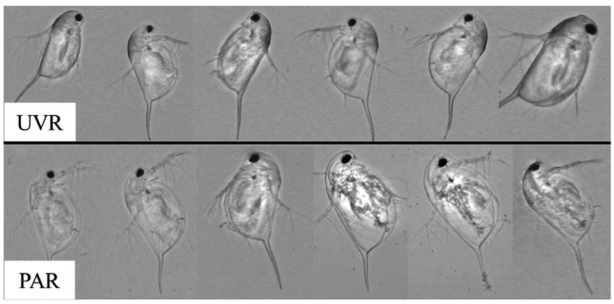

FIGURE 4 Photographs of *Daphnia dadayana*, on the sixth day of experimentation showing the differences in melanin at the back-neck zone. PAR, photosynthetic active radiation treatment; UVR, ultraviolet treatment

3.2 | Antipredator defenses under UVR

Neonates born in the flasks with the chemical signal of *P. sarsi* presented helmet heads (Figure 7), which represents a 5% in length increase. We also observed an enlargement of the caudal spine in the exposed animals. The average caudal spine length in induced animals was 0.58 ± 0.02 mm while that in animals without predator was 0.43 ± 0.03 mm. The total somatic length difference between animals exposed to *P. sarsi* versus control was around 25% (*t* test; $t = 15.753$, $GL = 27$, $p < .001$; Figure 8). The mean difference of total length in *D. dadayana* juveniles exposed versus control was 1.25 ± 0.04 mm versus 0.99 ± 0.05 mm (Figure 8).

In the experiment of UVR exposure of *D. dadayana* with versus without antipredator defense, there were no significant differences in GST activity. In addition, for the obtained values of GST activity,

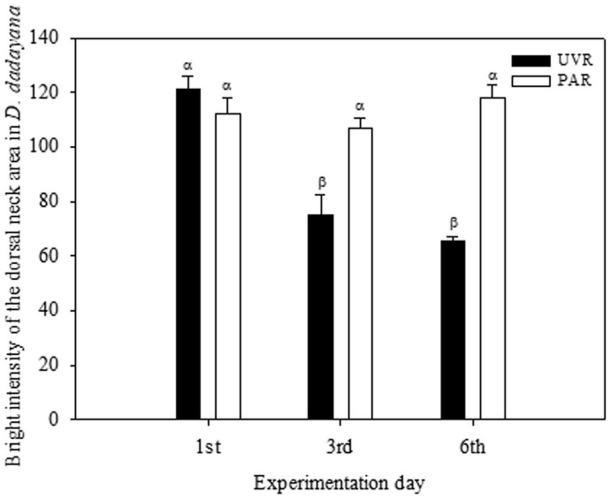

FIGURE 5 Results for bright intensity in the neck area of the carapace of *Daphnia dadayana* exposed to UVR. Bright intensity under both light conditions (black bars = PAR light and white bars = UVR light) for 6 days. Error bars represent the standard error and Greek symbols the significant differences. PAR, photosynthetic active radiation treatment; UVR, ultraviolet treatment

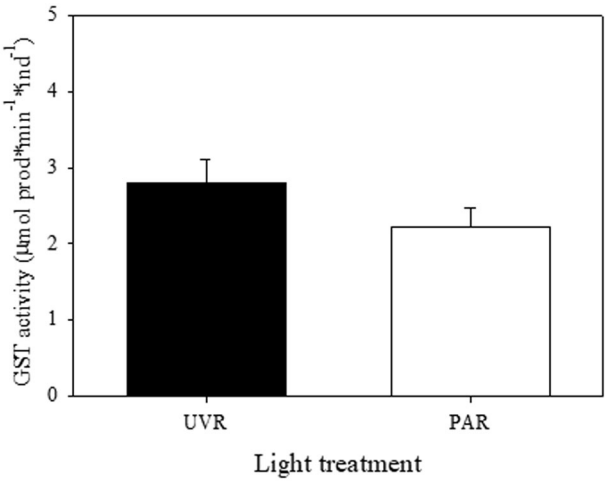

FIGURE 6 Results from *Daphnia dadayana* exposure to UVR versus PAR light. GST activity under two light conditions (black bar = PAR and white bar = UVR). Error bars correspond to the standard error. GST, glutathione S-transferase; PAR, photosynthetic active radiation treatment; UVR, ultraviolet treatment

we did not find an interaction between light and defense in the two-way ANOVA (two-way ANOVA defense × light $F_{1,12} = 0.00656$; $p = .937$; defense $F_{1,12} = 0.00265$; $p = .960$; light $F_{1,12} = 1.777$; $p = .207$; Figure 9). The analysis showed that there are no significant differences between UVR versus PAR and antipredatory defenses versus without defenses.

4 | DISCUSSION

We observed that melanic *D. dadayana* did not show an increase in GST activity when compared to the non-melanic exposed only to PAR. However, transparent *Daphnia* species from Patagonia increase GST activity under UVR exposure (Balseiro, Souza, Modenutti, & Reissig, 2008; Souza et al., 2007; Wolinski, Modenutti, Souza, &

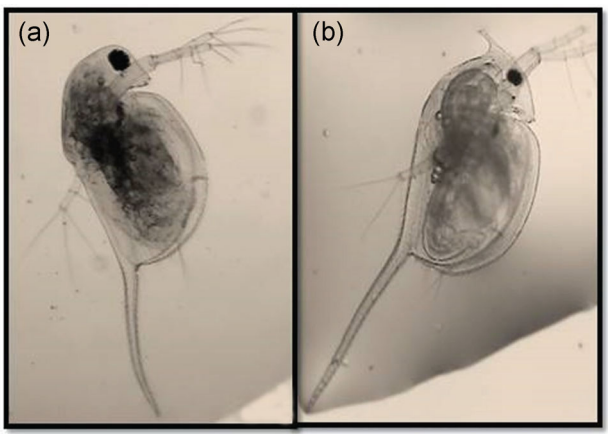

FIGURE 7 *Daphnia dadayana* neonate's photographs: (a) without defense induction and (b) with induced defense

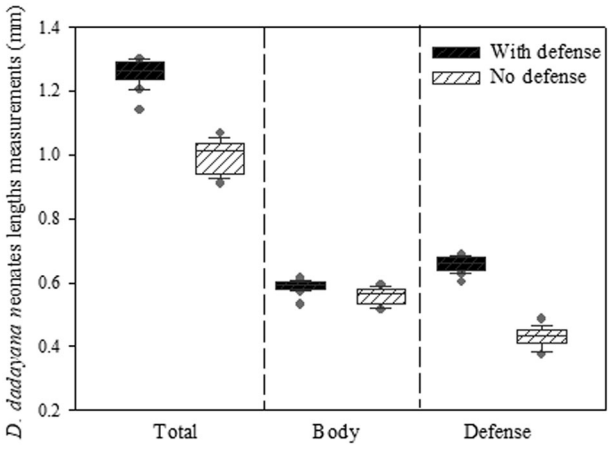

FIGURE 8 *Daphnia dadayana* length measures in millileters. Induced animals (black boxes with line pattern) versus control (white boxes with line pattern). Total length, body length, and defense length (including spine and helmet when induced)

Balseiro, 2016). Thus, melanin appears to be an efficient pigment shield especially in ponds with low dissolved organic matter (Hessen, Borgeraas, & Orbaek, 2002). In addition, the antioxidant we analyzed (GST) acts as a detoxifier, catalyzing ROS byproducts after the free radicals already reacted with organic molecules (Hayes & Pulford, 1995). Therefore, it seemed possible that melanin was enough for UVR protection; thus, the activation of a detoxifier enzyme, such as GST, was not required. However, because we measured only on the last day of the experiment we cannot reject the possibility that GST works while melanin is being synthesized by *Daphnia*. We also observed that melanin accumulation increases with increasing UVR exposure. Melanin accumulation in certain *Daphnia* species is a phenotypic feature that gives an advantage over other

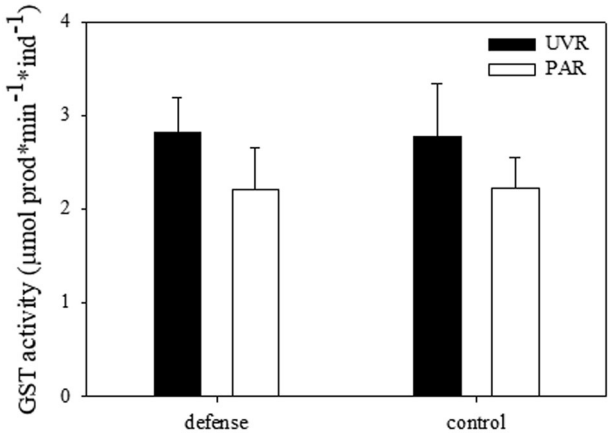

FIGURE 9 GST activity under two light conditions (black bars = UVR and white bars = PAR) and two defense stages (with defense and control without defense) on the third day of growth. Error bars represent the standard error. GST, glutathione S-transferase; PAR, photosynthetic active radiation treatment; UVR, ultraviolet treatment

species without pigments (Herbert & Emery, 1990; Hessen & Lydersen, 1996; Hessen et al., 1999; Zellmer, 1995), because pigmented animals can exploit surface layers to feed during sunlight hours (Zellmer, 1995).

The increase in melanin concentration due to a chronic UVR exposure of *D. dadayana* was successfully measured by image analysis. Melanin accumulation in the carapace is not uniform and its maximal concentration is in the dorsal neck area (Herbert & Emery, 1990). Image analysis has the advantage that the same alive individual can be used for different measurements along the experiment, while the chemical method is destructive and needs more animals to be killed at any time of the experiment. Furthermore, chemical extraction of melanin was observed to be inefficient, as a great proportion of the pigment remains in the carapace (Flössner, 1993; Herbert & Emery, 1990).

Here, we were able to demonstrate that the presence of the helmet and the enlargement of the caudal spine are induced by the presence of the predaceous copepod *P. sarsi*. Lateral photographs' analysis of the animals exposed to predator versus without, allowed us to measure a 25% increase in the size of newborns' total length. This increase in size will, in turn, give *D. dadayana* juveniles to have an extra chance to escape from *P. sarsi* attacks as was observed for other *Daphnia* species in Patagonia (Balseiro & Vega, 1994). Shallow lakes like Laguna Los Juncos lack a vertical light refuge for hazardous wavelengths (Burks, Lodge, Jeppesen, & Lauridsen, 2002). In contrast, in deep lakes with fish, the only strategy to avoid UVR effects is to perform diel vertical migrations (Williamson et al., 2011), as colored animals are easier to prey by visual fish predators. Here, we showed that *D. dadayana* is a resistant species to UVR suggesting that it is able to colonize shallow lakes with success. Furthermore, we observed that the investment in a antipredatory structure as helmet and spines did not affect this resistance. The lack of differences in GST activity in *D. dadayana* exposed to UVR observed in our experiment can be also due to the food conditions. Mothers were always fed ad libitum in this experiment (both in quantity and quality) and neonates were born with the helmet structure and the enlarged caudal spine under these conditions. As low food quality may also impose a weaker antioxidant response in *Daphnia* (Balseiro et al., 2008), it is likely that these well-fed animals did not show different enzymatic responses when exposed to UVR due to their rich nutrition. However, this situation does not necessarily occur because periods of food shortage are common in nature (Urabe et al., 2002). On the other hand, the obtained *p* values in GST analysis were high, meaning there are no significant differences or even incipient significance. Although the number of replicates were low (four) and a higher number of replicates could contribute to more accurate results, the change to significant differences does not seem likely.

Accumulating pigments in the carapace seems to be a successful and key strategy for *D. dadayana* in the shallow lakes it inhabits. Furthermore, *D. dadayana* is able to coexist with predators such as *P. sarsi* developing antipredation defenses that do not affect the antioxidant response when well fed. In the studied area, it has been predicted that under drier conditions, dissolved organic matter will be affected by higher photobleaching (Queimaliños et al., 2019), this condition will increase the UVR exposure in ponds like Los Juncos. In addition, predators can change due to species introductions that will affect, in turn, zooplankton diversity (Reissig, Trochine, Queimaliños, Balseiro, & Modenutti, 2006). In the present study, we showed that the resistance to UVR of the melanic *D. dadayana* is not affected by the investment in antipredatory defenses.

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