Life at the edge: Benthic invertebrates in high altitude Andean streams

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Chapter 4

Persistence of chironomids in metal polluted Andean high altitude streams: does melanin play a role?

Abstract

In high altitude Andean streams an intense solar radiation and coinciding metal pollution allow the persistence of only a few specialized taxa, including chironomids. The aim of the present study was therefore to determine the mechanisms underlying the persistence of chironomids under these multiple stress conditions, hypothesizing that melanin counteracts both the adverse effects of solar radiation and of metals. Melanin was determined in chironomids from reference and metal polluted streams at 3000 and 4000 m altitude, being two-fold higher at 4000 m compared to 3000 m, and two-fold higher in polluted streams than in reference streams at both altitudes. The field observations were experimentally verified by assessing the combined effects of Cu and UV-B on the survival and melanin concentration in larvae of the model species *Chironomus riparius* (Chironomidae, Diptera). In laboratory exposures, the highest melanin concentrations were found in larvae surviving toxic Cu concentrations, but not in those exposed to the highest UV-B radiation. Pre-exposure to UV-B decreased the sensitivity of the larvae to UV-B and to Cu+UV-B. It is concluded that in the field, melanin may protect chironomids partially against both elevated metal concentrations and solar radiation, allowing them to persist under the harshest conditions in high altitude streams.

Introduction

The tropical Andes encompass vast areas with altitudes above 4000 m, creating harsh environmental conditions, such as variable water temperatures, low oxygen levels and a blistering solar radiation, that challenge the survival and persistence of aquatic biota (Cabrera et al., 1997; Jacobsen and Marín, 2007; Jacobsen, 2008). Metal pollution may add further stress to life at high altitude, since in the Andes metals are continuously released by acid mine drainage and natural weathering of metal-rich bedrock (Smolders et al., 2003; Loayza-Muro et al., 2010). The few studies evaluating the effects of increased acidity and metal concentrations in high altitude tropical (van Damme et al., 2008; Loayza-Muro et al., 2010) and temperate streams (Courtney and Clements, 2000) have shown a reduction of invertebrate abundance and sensitive taxa richness, and a significant shift in community composition towards more tolerant taxa (Gerhardt et al., 2004).

Andean high altitude streams above 3500 m are also exposed to intense ultraviolet radiation, due to a naturally thinner ozone layer over low latitudes and the more direct solar light incidence near the equator (Kinzie et al., 1998). Especially UV-B (280–320 nm) influences the structure and functioning of aquatic communities by inhibiting plant production (Kinzie et al., 1998), and altering the abundance (Kiffney et al., 1997) and distribution of sensitive invertebrate species, either directly or indirectly by influencing trophic interactions (Kelly et al., 2003). The effects of UV-B are especially prominent during summer months, when clear skies and low water levels render benthic communities more vulnerable to solar radiation.

Benthic macroinvertebrates have evolved several defense strategies to reduce the damage caused by solar UV-B, including the accumulation or synthesis of photoprotective pigments, such as melanin, carotenoids and mycosporine-like amino acids (Krol and Liebler, 1998; Tartarotti et al., 2001; Persaud et al., 2007; Sommaruga, 2010). Melanin is a broad spectrum pigment, produced de novo by animals, which absorbs UV-B directly and releases the excess of energy as harmless heat (Hansson and Hylander, 2009; Sommaruga, 2010). Recently, it was discovered that in vitro melanin has the capacity to sequester reactive metal cations, such as copper, zinc and iron (Gallas et al., 1999; Szpoganicz et al., 2002; Hong and Simon, 2007), which are also key regulatory factors for its biosynthesis (Di Donato et al., 2002). Thus, the available evidence suggests that melanin counteracts both the adverse effects of solar radiation and metal toxicity, and may therefore be effective in fauna exposed to both stressors.

Under the harshest environmental conditions, polluted sites at the highest altitude in the Andes, chironomids were among the few persisting species (Loayza-Muro et al., 2010). Therefore, chironomids provide a unique test case to study how invertebrates cope with these multiple stressors, hypothesizing that melanin counteracts both the adverse

effects of solar radiation and metal pollution. To validate this hypothesis, melanin content was determined in chironomids from reference and metal polluted Andean streams at 3000 and 4000 m above sea level (m a.s.l.). The field observations were experimentally verified in laboratory tests, assessing the single and combined effects of Cu and UV-B on survival and melanin concentration in larvae of the model species *Chironomus riparius* (Chironomidae, Diptera).

Materials and Methods

Field sites

Eight sites were sampled in the Peruvian Andes, four being located in the Quilcayhuanca catchment and four in the Rúrec catchment, both in the Cordillera Blanca (Figure 1). In both catchments two clean and two polluted sites were located at respectively 3000 and 4000 m a.s.l. All sites were sampled in November (rainy season) 2009 and in July (dry season) 2010.
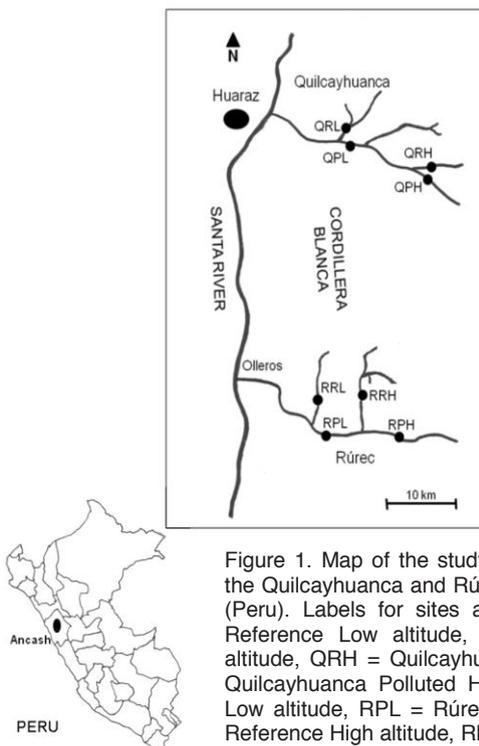
Figure 1. Map of the study area, indicating the sampling sites in the Quilcayhuanca and Rúrec catchments in the Cordillera Blanca (Peru). Labels for sites are as follows: QRL = Quilcayhuanca Reference Low altitude, QPL = Quilcayhuanca Polluted Low altitude, QRH = Quilcayhuanca Reference High altitude, QPH = Quilcayhuanca Polluted High altitude, RRL = Rúrec Reference Low altitude, RPL = Rúrec Polluted Low altitude, RRH = Rúrec Reference High altitude, RPH = Rúrec Polluted High altitude.

Physical and chemical parameters

Measurements of water pH, temperature (C°), conductivity and dissolved oxygen were performed at each sampling site using a WTW Multi 340i instrument equipped with SenTix® 41-3, TetraCon® 325-3 and CellOx® 325-3 probes (Weilheim, Germany). Solar ultraviolet-B irradiance (280–315 nm) was measured at the water surface with a Delta Ohm HD 2302.0 photo-radiometer and a LP 471 UVB probe with a quartz cosine corrector (Padua, Italy). UV-B irradiance was measured every two days during July (dry season) and November (rainy season), from 10:00 h to 14:00 h under full sun conditions, and the maximum values of each two days were averaged. Water samples for total metals analysis were taken in triplicate at each sampling site with 1-L polypropylene bottles, preserved with 10 N HNO_3 and analyzed by ICP-ES (induced-coupled plasma emission spectroscopy; Varian Liberty 100, USA). This analysis allowed detecting the following metals: Ag, Al, As, Ba, Be, B, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Sn, Sr, Ti, Tl, V and Zn. Quality control was carried out by analyzing USEPA destruction blanks and reference material for water samples.

Invertebrate sampling

Chironomidae larvae were sampled by hand at 3000 m and 4000 m a.s.l. from gravel-pebble substratum and stones along the banks, using forceps and a white plastic tray. Animals were transported to the laboratory, sorted under a Zeiss Stemi DV4 stereomicroscope (Göttingen, Germany), and identified to the family and sub-family level using taxonomical keys (Roldán, 1996; Domínguez and Fernández, 2009).

Melanin analysis

Melanin extraction followed the method by Hebert & Emery (1990) and Hobaek & Wolf (1991) with slight modifications, which have been validated previously in other benthic macroinvertebrate species exposed to UV-B radiation (Rautio and Korhola, 2002; Hansson et al., 2007; Connelly et al., 2009). Field collected fauna was preserved in 95% ethanol. After washing the samples with deionized water, five to ten individuals of the same size and taxon were pooled in triplicate, dried at 40 °C for 48 h and weighed. Organisms were grinded with a plastic pestle, placed in a test tube with 2 mL 5 M NaOH and homogenized in an ultrasonic bath (Branson 5210) for 10 min until complete emulsification. The tubes were heated overnight at 60°C with H_2O_2 (10 µL, 3% aqueous solution) and then centrifuged at 7800 g for 1 min. Quantification of the extracted pigment in the supernatant was performed spectrophotometrically at 350 nm (Shimadzu UV-1601) against a blank containing 5 M NaOH and H_2O_2 (3% aqueous solution). The absorbance was related to concentration (µg/mL) by a reference curve made from synthetic melanin (Sigma M8631, St. Louis, Missouri, USA) in 5 M NaOH and 10 µL H_2O_2 (3% aqueous solution), and the concentration of the pigment was normalized to dry weight (dw).

Laboratory test organisms and culturing conditions

Attempts to initiate a laboratory culture with field sampled high altitude Chironomidae larvae at sea level were unsuccessful, probably due to the large differences in oxygen concentration in the water. For this reason we chose for the lowland model species *Chironomus riparius* (Chironomidae, Diptera). Fourth instar larvae were obtained from an in-house laboratory culture at the University of Amsterdam. The culture was maintained in constantly aerated glass aquaria containing quartz sand overlaid with Dutch Standard Water (DSW, deionised water with 200 mg/L $CaCl_2 \cdot 2H_2O$, 180 mg/L $MgSO_4 \cdot H_2O$, 100 mg/L $NaHCO_3$ and 20 mg/L $KHCO_3$; hardness is 210 mg as $CaCO_3$/L and pH 8.2 ± 0.2) at 20 ± 1 °C, 65% humidity and a 16:8 h light:dark photoperiod. The culture was fed a mixture of Trouvit (Trouw, Fontaine-les-Vervins, France) and Tetraphyll (Tetrawerke, Melle, Germany) in a 20:1 ratio.

Cu toxicity experiments

The 96 h LC50 for Cu was determined following OECD Guideline 219 (2004) with slight modifications. A 100 mg/L $CuCl_2$ stock solution was used to test the following nominal Cu concentrations: 0 (control), 25, 50, 100, 200, 400, 800 and 1600 µg/L. Each concentration consisted of three replicates with ten fourth instar larvae in glass beakers containing 100 mL of DSW without sediment, food or aeration. After 96 h the surviving larvae were counted and kept frozen for Cu and melanin analysis. Copper was chosen because of its occurrence at high concentrations in polluted high altitude Andean streams (Smolders et al., 2003; van Damme et al., 2008; Loayza-Muro et al., 2010) and it's well documented toxicity to benthic macroinvertebrates.

Combined Cu and UV-B experiments

The combined Cu and UV-B experiments were conducted in glass beakers containing 100 mL of DSW without aeration, food or sediment. Water temperature was kept at 18 °C and a 16:8 h light:dark regime was applied. Four treatments with three replicates each and ten fourth-instar larvae per replicate were assayed for 96 h: control (without Cu and UV-B), Cu (nominal 100 µg/L ≈ LC50), UV-B (1.75 W/m^2, Arcadia D3 UV basking-lamp 160 W) and the combination of Cu and UV-B. After 96 h of exposure the surviving larvae were counted and kept frozen for Cu and melanin analysis.

The experiment was repeated with larvae pre-exposed to UV-B for 96 h under the same conditions as in the combined Cu and UV-B experiments, to simulate the natural situation of long-term exposure of Andean streams to high solar radiation. To this purpose, larvae were transferred from the cultures into a glass beaker containing 800 mL of DSW, and exposed to 1.75 W/m^2 UV-B. Larvae were fed and water was aerated and kept at 18 °C.

Cu and melanin analysis

To determine the actual Cu concentrations in the water, 2 mL water samples per replicate per treatment were taken after 1 and 96 h of exposure. These were acidified with 20 μL 65% HNO_3. To determine the Cu concentrations in the larvae, one larva per replicate per treatment was placed in a 2 mL Eppendorf tube and freeze-dried overnight at -53 °C in a Scanvac CoolSafe™ freeze dryer. The larvae were then weighed, 200 μL 65% HNO_3 was added and the samples were placed in a 100 °C heat block for 2.5 h. Next, 100 μL 65% HNO_3 was added and after 1.5 h 2 mL demi-water was added. Copper concentrations were determined by flame atomic absorption spectrophotometry (Perkin-Elmer AAnalyst 100, Germany). The Cu concentrations in the water after 1 and 96 h of exposure were averaged for each replicate to calculate the actual Cu concentration. Quality control of copper analyses was carried out by analyzing destruction blanks and reference material for water (NIST SRM 1643d) and for larvae (IAEA MAA-3/TM shrimp homogenate). Measured values were in good agreement with certified values (< 10% deviation) and destruction blanks near detection limits.

To determine the melanin concentrations in the larvae, three individuals per replicate per treatment were pooled and processed as described for field collected animals.

Statistical analysis

To determine the LC50 for Cu the logistic response model $y=c/(1+e^{b(log(x)-log(a))})$, adopted from Haanstra et al. (1985), was fitted through the concentration-response data with y being survival, a the LC50, b the slope of the logistic curve, c the average survival in the control, and x the actual Cu concentration in the water. In the Cu toxicity experiment, the Cu concentrations in the larvae were compared using a one-way analysis of variance (ANOVA). A Bonferroni post-hoc test was conducted to determine significant differences between treatments. To determine the relationship between the melanin concentration in the larvae and the Cu concentration in the water, and between the melanin concentration in the larvae and the Cu concentration in the larvae, a Pearson product-moment correlation test was run.

A two-way ANOVA was applied to determine the effects of metal pollution, altitude and metal pollution+altitude on the melanin concentrations in the larvae sampled in the field, and whether there was an interaction between metals and altitude. For the multifactorial laboratory experiment also a two-way ANOVA was applied to determine the effects of Cu, UV-B and Cu+UV-B on survival, and on the melanin and Cu concentrations in the larvae, and whether there was an interaction between Cu and UV-B. A Bonferroni post-hoc test was conducted to determine significant differences between treatments. Independent samples t-tests were run to compare survival and melanin concentrations in UV-B pre-exposed larvae with non-pre-exposed larvae. In all cases, data were log-

transformed when necessary to meet variance homogeneity. All tests were run in SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) and a significance level of $p < 0.05$ was applied.

Results

Physical chemical characteristics

The UV-B radiation level at 4000 m was two-fold higher than at 3000 m both in the dry and rainy seasons, while water temperature and dissolved oxygen were similar between altitudes and seasons (Table 1). Conductivity was higher and pH was lower in the polluted streams compared to the reference streams. Likewise, the concentrations of all metals were higher in the polluted streams than in the references streams, and increased from the rainy to the dry season (Table S1). The metal concentrations at the polluted sites (e.g. Al, 4.83 mg/L; As, 0.025 mg/L; Cu, 0.197 mg/L; Fe, 58.8 mg/L; Mn, 1.17 mg/L; Ni, 0.11 mg/L; Zn, 0.256 mg/L) ranged from 6 (As and Sr) to 588 (Fe) times those at the reference sites. At these polluted sites, the streambed was smothered by orange precipitates and encrusted layers, most likely dominated by iron oxyhydroxides.

Table 1. Physical and chemical variables at the eight sampling sites in the Cordillera Blanca area, Peru. Status: Ref, reference; Poll, polluted.

Catchment	Season	Altitude	Status	UV-B (W/m^2)	Conductivity (µS/cm)	pH	Temperature (°C)	Oxygen (mg/L)
Quilcayhuanca	Dry	3,040 m	Ref	2.11	73	6.5	14.0	5.2
			Poll	1.92	155	5.2	14.2	5.4
		3,998 m	Ref	4.89	70	7.4	13.4	5.6
			Poll	4.92	219	4.3	14.1	5.5
	Rainy	3,040 m	Ref	1.08	29	7.3	10.0	5.9
			Poll	1.03	125	4.6	10.9	5.8
		3,998 m	Ref	3.04	52	7.5	8.7	6.0
			Poll	2.95	179	4.2	12.4	5.1
Rúrec	Dry	3,087 m	Ref	2.21	60	7.3	13.1	5.4
			Poll	2.06	279	5.3	13.5	5.3
		4,079 m	Ref	5.23	56	6.6	10.6	5.1
			Poll	5.09	738	3.2	10.6	5.3
	Rainy	3,087 m	Ref	1.23	34	6.8	11.0	5.6
			Poll	1.18	138	4.1	10.0	5.5
		4,079 m	Ref	2.99	41	6.4	12.3	4.8
			Poll	3.13	320	3.5	8.4	4.9

Melanin in field collected chironomids

Chironomid larvae belonging to the sub-family Chironominae were densely pigmented at the highest sampling sites, and accordingly the melanin levels in larvae from reference streams at 4000 m were 2.5-fold higher (70.6–75.1 µg/mg dw) than in larvae from 3000 m (27.5–30.8 µg/mg dw) (Figure 2), thus showing a significant effect of altitude ($p < 0.001$). At both altitudes, melanin was two-fold higher in larvae from polluted streams than in larvae from the reference streams, revealing a significant effect of metal pollution ($p < 0.001$). Consequently, the highest melanin concentrations (136.5–158.3 µg/mg dw) were measured at the polluted high altitude sites, although there was no interaction between metal pollution and altitude ($p > 0.05$).

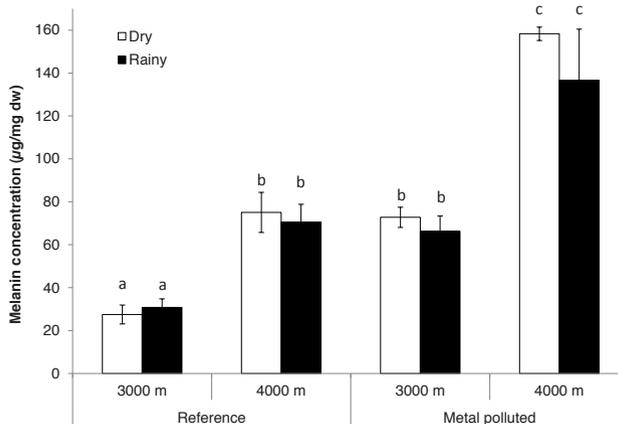Figure 2. Melanin levels (average ± s.e.) in field collected chironomids (µg/mg dw) from reference and polluted sites at 3000 and 4000 m a.s.l. during the dry and rainy seasons. Ref, reference; Poll, polluted; Letters denote significant differences ($p < 0.05$) in melanin concentration between pollution status and altitude.

Cu toxicity

The actual Cu concentrations in the water for the Cu toxicity experiment were 14 (control), 23, 63, 132, 259, 476, 900 and 1503 µg/L, and control survival was 77%. A clear dose-response relationship was observed for the effect of Cu on larval survival after 96 h of exposure (Figure 3A). From the logistic response model the LC50 was calculated to be 80 µg/L (95% CL:71–89). There was a significant increase ($p < 0.001$) and positive correlation for both Cu ($n = 3$, $r = 0.98$, $p = 0.001$; Figure 3B) and melanin ($n = 3$, $r = 0.95$, $p = 0.009$; Figure 3C) concentrations in the surviving larvae with increasing water Cu concentrations.
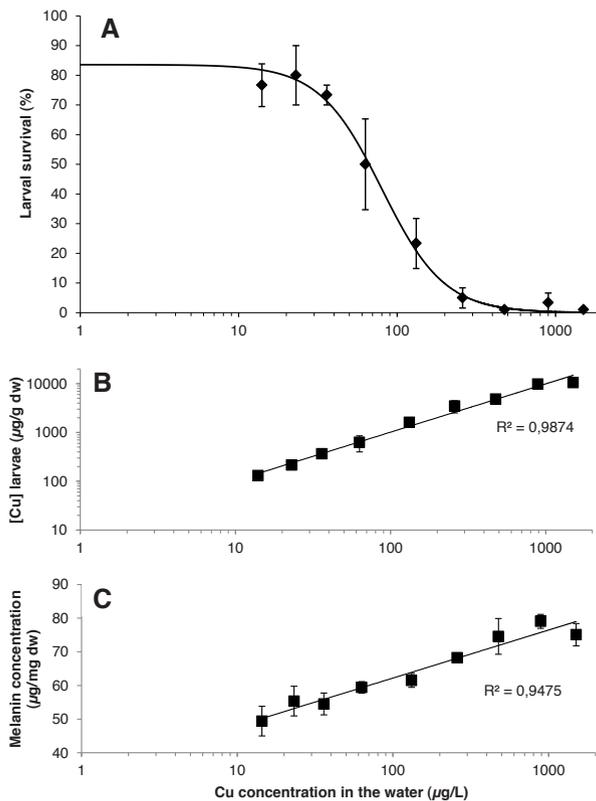
Figure 3. Survival (**A**, average % ± s.e., n = 3), body Cu (**B**, average ± s.e., n = 3; µg/g dw) and melanin concentration (**C**, average ± s.e., n = 3; µg/mg dw) in C. riparius larvae after 96 h of exposure to different Cu concentrations in the water (µg/L). The line in A indicates the logistic response model of Haanstra et al. (1985). For melanin and internal Cu concentrations the regression coefficients are shown.

Combined effects of Cu and UV-B

The actual Cu concentrations in the water were 14 (control), 3 (UV-B), 76 (Cu) and 83 (Cu+UV-B) µg/L, and control survival was 78%. For the 96 h UV-B pre-exposure experiment, these were 1 (control), 3 (UV-B), 72 (Cu) and 76 (Cu+UV-B) µg/L, and control survival was 87%. For the non-pre-exposed larvae significantly lower survival was observed in the Cu ($p < 0.05$), UV-B ($p < 0.001$) and Cu+UV-B treatments ($p < 0.001$) compared to the control, the Cu+UV-B treatment showing the lowest survival (Figure 4A). The two-way ANOVA indicated that this was caused by significant main effects of both Cu ($p < 0.01$) and UV-B ($p < 0.001$), but not by their interaction. Pre-exposure to UV-B led to
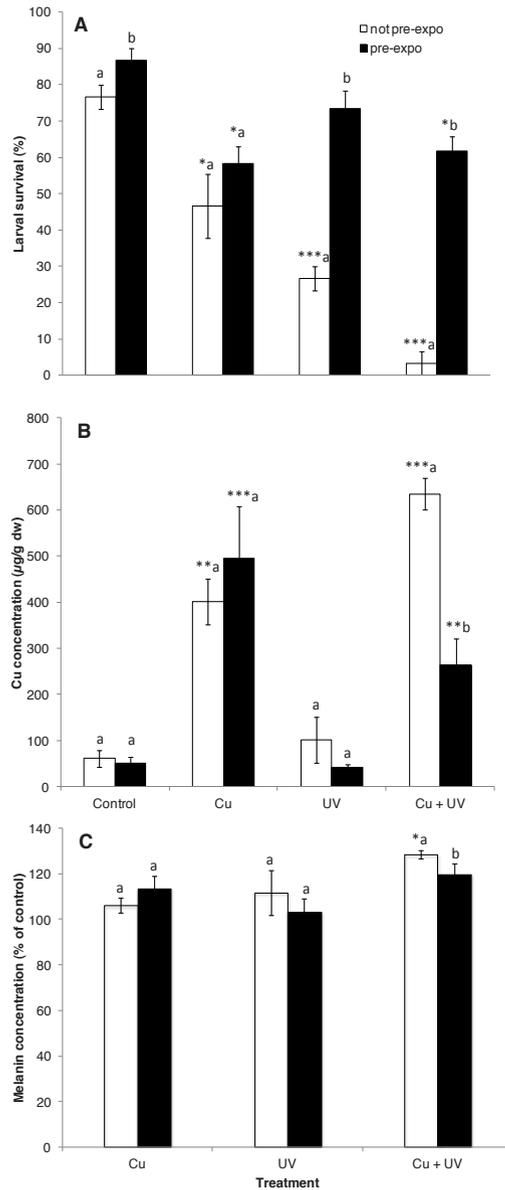
Figure 4. Survival (**A**, average % ± s.e., *n* = 3), body Cu concentration (**B**, average ± s.e., *n* = 3; µg/g dw) and body melanin concentration (**C**, average % of control ± s.e., *n* = 3) in the single exposures to Cu and UV-B, and in the combined Cu + UV-B treatments with and without 96 h UV-B pre-exposure. Asterisks indicate values significantly different from controls, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Letters denote significant differences between the non-UV-B pre-exposure and UV-B pre-exposure treatments.

significantly higher survival in larvae exposed to UV-B ($p < 0.001$) and especially to Cu+UV-B ($p < 0.001$) compared to the non-pre exposed larvae, and to similar survival of the UV-B and the control treatments (Figure 4A).

The Cu concentrations in non-pre-exposed and UV-B pre-exposed larvae were significantly higher in the Cu ($p < 0.01$) and Cu+UV-B ($p < 0.001$) treatments compared to the control. The non-pre-exposed larvae contained the highest Cu concentration after Cu+UV-B exposure, which was significantly higher than after the exposure to Cu alone (Figure 4B) coinciding with the lowest larval survival (Figure 4A). This was caused by positive main effects of both Cu ($p < 0.001$) and UV ($p < 0.05$), but not by their interaction ($p > 0.05$). Pre-exposure to UV-B produced significantly less Cu accumulation in larvae exposed to Cu+UV-B compared to Cu alone and to non-pre-exposed larvae ($p < 0.05$; Figure 4B), and coincided with a strong increase in larval survival (Figure 4A).

A small but significant increase in melanin concentration was only observed in the Cu+UV-B treatment ($p < 0.05$) caused by a main effect of UV ($p < 0.05$), but not by the interaction between Cu and UV ($p > 0.05$; Figure 4C). Pre-exposure to UV-B did not result in higher melanin concentrations in any of the treatments compared to the control and to the non-pre-exposed larvae (Figure 4C).

Discussion

High up in the Andes polluted streams are exposed to high metal mixture concentrations and an extreme UV-B radiation, which may create unique multistress conditions allowing the survival of only few specialized taxa, including chironomids. The aim of the present study was therefore to determine the mechanisms enabling these chironomids to persist in this harsh environment, hypothesizing that melanin counteracts both the adverse effects of solar radiation and metal pollution. Chironomids from metal polluted high altitude streams indeed contained twice as much melanin as those from reference sites at the same altitude, although this was not a result of the interaction of altitude and metal pollution, but of their independent effects. This may represent a protective mechanism against toxic metals given the ability of melanin to bind and sequester reactive metal cations, also mitigating their damaging potential as inducers of oxidative stress (Gallas et al., 1999; Hong and Simon, 2007). An attempt to validate this hypothesis was conducted by transplanting field populations across the 4 sites to assess the effects of metals and altitude on survival and melanin content. However, this was not possible since chironomids from reference sites did not survive transplantation to polluted streams at both altitudes, nor did those transplanted from 3000 to 4000 m a.s.l. Moreover, to corroborate these observations under laboratory conditions, attempts were made to

initiate a culture with field sampled high altitude chironomid larvae at sea level. However, since this was unsuccessful, probably because of the large differences in environmental conditions, such as the higher oxygen concentration in the water at sea level, we chose for the lowland model species *Chironomus riparius* belonging to the same subfamily (Chironominae) as those sampled in the field. Although this may have come with some limitations and Andean populations are likely to be genetically adapted to their environment, our laboratory experiments confirmed the field observations: in the Cu toxicity experiments the few larvae surviving the highest Cu exposures, and containing the highest Cu body concentrations, also showed the highest melanin body concentrations, suggesting that high melanin concentrations may convey Cu tolerance in cultured chironomid larvae. This was also evident for the combined Cu and UV-B treatment, survived by very few larvae that showed high internal Cu concentrations and slightly elevated melanin levels, but not for the UV and Cu treatments alone. Exposure to Cu did not increase melanin levels, more likely because the tested Cu concentration, the 96 h LC50 (80 μg/L) was much lower than those in the Cu toxicity test (132–1503 μg/L) survived by highly melanized larvae. These results do not necessarily indicate that melanin is the only mechanism conferring tolerance to UV-B and metal pollution. Melanin may act together with other defenses, such as metallothioneins, glutathione and antioxidant enzymes, such as superoxide dismutase, catalase and glutathione-S-transferase, which may covary similarly as pigmentation (Meng et al., 2009).

The laboratory experiments also confirmed the joint but non-interactive effects of multiple stressors, suggesting that in high altitude Andes the even higher metal concentrations and UV-B irradiances may act independently, likely causing challenging conditions for the persistence of aquatic biota. Given the clear differences among our test sites in the Andes, this detrimental effect might be more pronounced during the dry season, when shallow depths and low DOM content in the water render chironomids more exposed to solar radiation (Kelly et al., 2001; Kashian et al., 2004; Loayza-Muro et al., 2010). These similar but independent effects have been observed in other elevated metal polluted streams, where a replacement of sensitive taxa by metal-tolerant chironomids occured (Clements, 1994; Kiffney and Clements, 1994), and suggests that UV-B exposure may increase the susceptibility of macroinvertebrates to metal toxicity or vice versa (Kashian et al., 2007).

Melanin concentrations in chironomids from reference streams increased with increasing UV-B levels measured at 3000 and 4000 m in the Andes. This may represent a defense mechanism against the damaging effects of intense solar radiation as described in previous studies on melanin, mycosporine-like amino acids and carotenoids in copepods and cladocerans at high altitudes (Helbling et al., 2002; Sommaruga, 2010) and in arctic habitats (Rautio and Korhola, 2002; Rautio and Bonilla, 2009). Melanin field observations

were corroborated in larvae exposed to UV-B in the laboratory, although it caused only a slight increase in melanin concentrations. This could be explained by the relatively low intensity of the UV lamp (1.75 W/m^2) compared to the highest sunlight regimes at 4000 m in the Andes (5.23 W/m^2).

Pre-exposure to UV-B for 96 h resulted in a lower sensitivity to the UV-B and Cu+UV-B treatments, although it did not increase the melanin concentrations in any of the treatments compared to the non-pre-exposed larvae. This suggests that melanin is not the exclusive protective mechanism against Cu and UV-B, and hence other photopigments or enhanced free radical scavenging and antioxidant capacity could be involved (Meng et al., 2009). Pigments like carotenoids and mycosporine-like amino acids are known adaptations to harsh UV-B conditions (Sommaruga, 2010) and thus possible candidates for causing the observed lower sensitivity to UV-B of pre-exposed larvae. However, these pigments are not synthesized by animals, implying that the larvae would have acquired them from their food in the culture given the short duration of the laboratory experiment. A lowered sensitivity could also be explained by a lower Cu accumulation. The incorporation of Cu observed in larvae during the laboratory experiments must have occurred by passive uptake through the gut epithelium, body orifices or surface adsorption, which are the most likely mechanisms for metal uptake in insect larvae (Hare, 1992; Timmmermans et al., 1992). Hence, the reduced Cu accumulation observed in larvae exposed to Cu+UV-B after UV pre-exposure was most probably due to a decreased permeability to Cu produced by sclerotization, likely stimulated by a prolonged UV-B exposure period consisting of pre-exposure to UV-B and exposure to UV-B in the Cu+UV-B treatment. In contrast, the Cu only treatment received UV during the pre-exposure, but not during the actual experiment (Sugumaran, 2002). These mechanisms, and not an increase in melanin concentration, may have diminished the detrimental effects observed in the combined Cu and UV-B experiment. Although melanin may partially convey tolerance in chironomids persisting for long periods under metal and UV-B exposure in the high Andes, melanin-mediated tolerance was not indicated in the laboratory experiments as the key mechanism for short-term responses to Cu and especially to UV-B. The observed responses of *C. riparius* may be based on its plasticity to cope with a wide variety of stressors, such as organic pollutants (Gower and Buckland, 1978; Friberg et al., 2010), metals (Havas and Hutchinson, 1982), acidity (Jernelöv et al., 1981), salinity (Bervoets et al., 1996) and anaerobic conditions (Redecker and Zebe, 1988), and on its rapid growth and short life cycle (Groenendijk et al., 1998).

Supporting information: Table S1. This information is available free of charge via the Internet at http://pubs.acs.org.