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

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Chapter 5

UV-B-driven pigmentation and genetic diversity of benthic macroinvertebrates from high altitude Andean streams

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Abstract

Photoprotective pigments in benthic macroinvertebrates may reduce the damage caused by the blistering UV-B radiation in Andean high altitude streams above 3500 m. The aim of the present study was therefore to determine if melanization in macroinvertebrates inhabiting high altitude Andean streams is an adaptive response to high UV-B radiation. To explore if altitude-related differences in melanin concentration between taxa were due to a variable community composition or to population differentiation, mayfly species were identified genetically.

We measured UV-B radiation from 650 to 4000 m and compared body melanin concentrations from several benthic macroinvertebrate orders sampled at these altitudes. Five genera belonging to the mayfly family Baetidae were genetically identified to the species level. DNA sequencing was performed in individual larval legs to group genetically similar individuals before pigment analysis in the corresponding bodies.

The UV-B radiation at 4000 m was twice that at 3200 m, four-times that at 1900 m and five-times that at 650 m. The melanin concentration in families belonging to Ephemeroptera, Trichoptera, Diptera and Turbellaria was twice as high at 4000 m as at 3200 m, but did not differ among taxa or between seasons. Five genera of the family Baetidae were identified: *Americabaetis*, *Dactylobaetis*, *Tupiara*, *Baetodes* and *Thraulodes*. Genetic differences were evident between *Americabaetis* sp. at 4000 m from the Cordillera Blanca and at 3200 m from the Rímac River valley, and between *Tupiara* taxa at 650 and 1900 m in the Rímac River. In *Americabaetis* melanin increased five-fold from 1900 to 4000 m, while in *Dactylobaetis* and *Tupiara* it was twice as high at 1900 m as at 650 m. In *Baetodes* melanin at 4000 m was twice that at 650 and 1900 m, while in *Thraulodes* it was almost three times higher at 4000 m than at 3200 m.

In *Tupiara*, the differences in melanin levels were probably associated with species with different vertical distribution, while in *Dactylobaetis* these differences were interpreted as phenotypic plasticity. Our results thus indicate that mayfly species within a single family have both constitutive or adjustable melanin concentrations, enabling them to cope with the strong selective UV-B environment. Adjustable melanin levels have commonly been observed under moderate UV-B regimes, while the constitutive, high melanin concentration is probably an attribute of high altitude invertebrate fauna in the tropics.

Introduction

Species inhabiting high altitude aquatic ecosystems are exposed to harsh environmental conditions, such as low water temperature, low oxygen concentration and high solar radiation, which challenge the survival and persistence of aquatic biota (Cabrera et al., 1997; Sommaruga, 2001; Jacobsen and Marin, 2007; Jacobsen, 2008). Andean high altitude aquatic ecosystems above 3500 m may be particularly exposed to intensive ultraviolet-B (UV-B) radiation, due to the thinner ozone layer over low latitudes and the shorter path of solar radiation through the atmosphere near the equator (Villafañe et al., 1999). This high UV-B exposure is accentuated by a sparse riparian canopy that provides little shade and by the very clear water. The concentration of dissolved organic matter (DOM), the principal constituent absorbing solar radiation (including UV) in fresh waters, is low (Laurion et al., 2000; Kelly, Clare and Bothwell, 2001; Clements et al., 2008). Nevertheless, little attention has been devoted to the effects of UV-B radiation on macroinvertebrate diversity and community composition in high altitude Andean streams (Cabrera, et al., 1997; Tartarotti et al., 1999, Loayza-Muro et al., unpublished).

Natural ultraviolet-B radiation (UV-B, 280–320 nm) may influence the structure and function of aquatic communities by inhibiting primary production (Kinzie et al., 1998), altering the abundance and diversity of the biota (Kiffney et al., 1997a), limiting the distribution of sensitive species, influencing trophic interactions (Kelly et al., 2003) and damaging DNA (Macfadyen et al., 2004). At high altitudes in the Andes (>3500 m), the maximum UV-B values in the dry season (5.23 W/m^2, Loayza-Muro et al., 2013) are well above those causing significant drift and mortality of invertebrates in artificial streams and structuring natural invertebrate communities in other mountainous areas (Kiffney et al., 1997a, Kiffney et al., 1997b). Indeed, the differences observed in benthic community composition along an altitude gradient between 2000 and 4000 m have been attributed to this increased UV-B radiation and low DOC content in the streams (Loayza-Muro et al., unpublished), suggesting species-specific sensitivities towards high UV-B conditions (Cabrera et al., 1997; Tartarotti et al., 2001; Marinone et al., 2006).

Planktonic crustaceans have evolved several defence strategies to reduce the damage caused by natural solar UV radiation, including behavioural escape responses (Rhode et al., 2001), photoenzymatic repair of DNA (Macfadyen et al., 2004), antioxidant defences (Souza et al., 2007) and photoprotective pigmentation (Hessen et al., 2002). In shallow clear fresh waters, especially those at high altitudes, one of the first lines of defence is the accumulation or synthesis of photoprotective pigments, such as melanin, carotenoids and mycosporine-like amino acids (MAAs) (Sommaruga and García-Pichel, 1999; Tartarotti et al., 2001; Hansson et al., 2007; Persaud et al., 2007; Sommaruga, 2010). These substances are both effective solar radiation screeners and antioxidants, providing

protection against detrimental photoproduced radicals (Hairston, 1979; Krol and Liebler, 1998).

Melanin is a broad spectrum tan-brown to black cuticular pigment with an absorption maximum between 250 and 350 nm that absorbs UV radiation directly and releases the excess energy as harmless heat (Sommaruga, 2010). Several studies have shown that melanic organisms can tolerate high solar irradiance better than non-pigmented relatives, and that the level of pigmentation is an inducible and adjustable defence mechanism (Hessen et al., 1999; Rautio and Korhola, 2002; Hansson, 2004, Hansson et al., 2007).

Photoprotective melanization is typically observed in *Daphnia* as well as in other cladocerans from arctic and alpine clear freshwater habitats exposed to UV-B radiation (Hebert and Emery, 1990; Rautio and Korhola, 2002; Rautio et al., 2009; Sommaruga, 2010), but has seldom been studied in benthic macroinvertebrates from high altitude streams. In the high Andes (> 3000 m), UV-B radiation presents a strong temporal and spatial variation, increasing with altitude and being particularly intense during the summer, when radiation peaks and reduced cloudiness and shallow water leave benthic communities more exposed to solar radiation (Cabrera et al., 1995; Zaratti, 2003; Loayza-Muro et al., 2013). Given these strong UV-B gradients, we hypothesized that pigment concentration in macroinvertebrates may be an adaptive response to particularly high UV-B radiation, especially during the dry season at the highest altitude sites. To test this hypothesis, we compared body pigment concentrations from several invertebrate taxa that were sampled during the dry and rainy season in streams ranging from 650 to 4000 m above sea level (a.s.l.) in the Peruvian Andes. To determine if potential differences in pigment concentration between individuals from different sites and seasons were due to a variable community composition (i.e. species turnover) or to population differentiation (i.e. interspecific differences), identification of the benthos to species was necessary. However, since conventional identification to species in this region is hampered by the availability of keys, DNA sequencing was performed in individual larval legs to group genetically similar individuals before pigment analysis in the corresponding bodies.

Methods

Study sites

Below the permanent snow-line, between 3700–4400 m a.s.l., Andean streams are fast flowing, with substrates consisting of gravel, pebble, cobbles and stones in runs and riffles at higher altitudes, and larger rocks at lower altitudes. They show transparent waters,

a very sparse macrophyte growth and are almost completely unshaded, particularly above 4000 m.

Five sites were sampled. Two sampling sites, one at 3200 m and the other at 4000 m, were selected in the Quilcayhuanca catchment in the Cordillera Blanca (Figure 1). In the Rímac River three sites were selected (at 650, 1900 and 3200 m). All sites were sampled in November (rainy season) 2009 and in July (dry season) 2010.

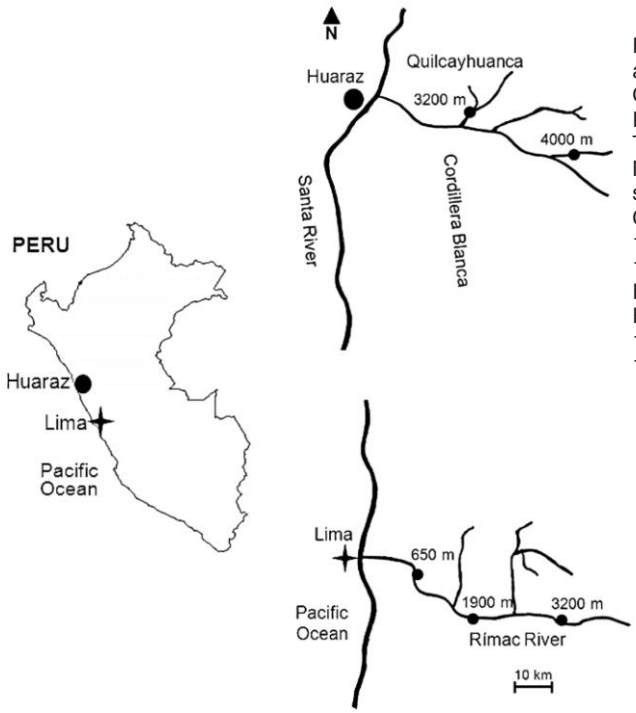

Figure 1. Map of the sampling area, indicating the sites in the Quilcayhuanca River and the Rímac River catchment, Peru. The UTM (Universal Tranverse Mercator) coordinates for the sampling sites are: Quilcayhuanca River 3200 m, 18224401E 8946087N; 4000 m, 18235408E 8949765N. Rímac River 650 m, 18305903 E 8675758N; 1900 m, 18341845E 8685832N; 3200 m, 18361932E 8702964N.

Physicochemical characteristics

Solar ultraviolet-B irradiance (280–315 nm) was measured at the water surface with a HD 2302.0 photo-radiometer and a LP 471 cosine-corrected broad-band UV-B sensor, with a maximum detection at 305 nm (Delta Ohm, Padua, Italy), and calibrated in October 2009 before field measurements. UV-B irradiance was measured every second day during November 2009 (rainy season) and July 2010 (dry season), from 10:00 h to 14:00 h under full sun, and the maximum values obtained during each month were averaged. Water samples for analysis of dissolved organic carbon (DOC) were filtered through 0.45-μm Whatman GF/F glass fibre filters (GF/F) in the field, acidified with HCl, and stored in amber glass bottles at 4°C. The concentration of DOC was measured as non-purgeable

organic carbon within 24 h using a TOC-5000 analyser (Shimadzu, Columbia, ML, U.S.A.). Calibration standards were prepared from dilutions of organic carbon primary standard in laboratory reagent water preserved to pH ≤ 2 with concentrated acid. Filter blanks for organic contamination were included. Each sample was analysed in triplícate. In addition standard abiotic factors including pH, temperature (C°), conductivity and dissolved oxygen were measured at each sampling site using a multi 340i instrument equipped with SenTix® 41-3, TetraCon® 325-3 and CellOx® 325-3 probes (WTW, Weilheim, Germany). Stream depth was calculated from four measurements at each of three parallel cross-sections with a calibrated stick.

Invertebrate sampling

Mayflies were sampled at 650, 1900, 3200 and 4000 m in streams with very sparse macrophyte growth. Caddisflies, blackflies, midges and flatworms were sampled only at 3200 and 4000 m because of their restricted distribution along the altitude gradient. Organisms were collected from gravel-pebble substratum and under stones along the banks, using forceps and a white plastic tray. Animals were transported to the laboratory, sorted under a Zeiss Stemi DV4 stereomicroscope, and identified to family using the keys of Roldán (1996) and Domínguez and Fernández (2009). Since mayflies were present over the whole altitudinal gradient, they were considered the most promising group to study at a finer taxonomic level, for which they were morphologically identified to genus and then to species by genetic analysis. For this purpose, two legs of each individual mayfly larva were separated in individual 1.5-mL tubes with 0.2 mL 95% ethanol, while the corresponding bodies were saved separately in 1.5-mL tubes with 95% ethanol for the determination of melanin concentration.

Genetic species identification

DNA was extracted from the legs of individuals that had been identified morphologically to genus, following the manufacturer's instructions contained in the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands). In the first step of extraction, the legs were placed in a 1.0-mL tube with 180 µL ATL buffer and zirconia beads (1.0 mm) and homogenized using a Precellys Tissue Homogenizer. After extraction, the presence of DNA was verified using a 1% agarose gel and the quantity and quality of the DNA extract was measured using a NanoDrop (ND-1000, Isogen Life Science) spectrophotometer. DNA extracts were then stored at -20°C.

For cytochrome oxidase I (COI) amplification, purification and sequencing, DNA samples were taken from -20°C and thawed on ice and, if necessary, diluted to a maximum concentration of 35 ng DNA/µL. An approximate 700-basepair portion of the COI gene was amplified using a Polymerase Chain Reaction (PCR), and the primers LCO1490 (forward, 5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (reverse, 5'-

TAAACTTCAGGGTGACCAAAAAATCA-3'), which have been used before in a broad range of invertebrates (Folmer et al., 1994). For this, 2.5 µL of each DNA sample was mixed with 17.5 µL of a PCR cocktail, consisting of 8.3 µL H_2O, 4.0 µL 5X PCR Buffer, 4.0 µL 1mM dNTPs, 0.5 µL 10 µM of each forward and reverse primer and 0.20 µL 5U/µL "Hot Start" Taq polymerase (Finnzymes, Finland). PCR cycling conditions were: initial denaturation at 98°C for 30 sec, 35 cycles of denaturation at 98°C for 5 sec, annealing at 48°C for 5 sec, extension at 72°C for 15 sec, a final extension step at 72°C for 60 sec and then cool down at 4°C for 300 sec. Quality and size of the PCR products was determined on a 1% agarose gel. PCR products were sent to MacroGen Europe (Amsterdam, Netherlands) for sequencing.

For species identification, phylogenetic and molecular evolutionary analyses of CO1 sequences were conducted using MEGA 5.0 (Tamura et al., 2011). First, Genbank was searched to confirm that our sample sequences were CO1 and determine the taxonomic identity of each sequence. Next, sequences were aligned using ClustalW and trimmed so that all sequences were of equal length. The phylogeny was inferred by using the Maximum Likelihood method and the best fitting nucleotide substitution model that was selected based on the lowest Bayesian information criterion. Based on the obtained phylogenetic tree, individuals belonging to the same taxa were pooled, allowing reliable quantification of the melanin concentrations in their bodies.

Melanin analysis

Melanin extraction was performed in triplicate for each taxon, following the method by Hebert and Emery (1990) and Hobaek and 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). After washing the samples in 95% ethanol with deionized water, three individuals of the same size in the case of mayflies, caddisflies and flatworms, and three pools of five individuals of the same size in the case of midges and blackflies were dried separately at 40°C for 48 h and weighed. Organisms were ground with a plastic pestle, placed separately in test tubes 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, free of residuals, was performed spectrophotometrically at 350 nm against a blank containing 5 M NaOH and H_2O_2 (3% aqueous solution). The absorbance was related to concentration (µg/mL) using a six-point reference curve (6.25–100 µg/mL) 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).

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine differences in macroinvertebrate melanin concentrations between sites differing in UV-B irradiance, and sites were compared with each other with Tukey's post hoc test. When necessary, data were log-transformed to meet variance homogeneity. Analyses were performed in SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Physicochemical characteristics

In the Quilcayhuanca River, UV-B radiation at 4000 m was twice that at 3200 m in both the dry and rainy seasons. The same two-fold difference was observed for sites at 3200 and 1900 m in the Rímac River, while there was a 1.5-fold difference between 1900 and 650 m (Table 1). This confirms the presence of an UV-B gradient along the sampling sites, with the highest UV-B radiation value (4.89 W/m^2) at the highest sites at 4000 m. Water pH ranged from 6.4 to 8.5, temperature from 9.7 to10.3°C, and dissolved oxygen concentrations from 4.8 to 6.1 mg/L throughout the study. Conductivity in the C. Blanca (29–73 µS/cm) was lower than in the Rímac River (168–479 µS/cm). The lowest DOC concentrations (0.52–0.72 mg/L) were measured at 4000 m during the dry season, increasing significantly at lower altitudes in both catchments. A slightly higher DOC concentration was found at 4000 m in the rainy season, and a similar increase towards lower altitudes.

Melanin concentrations in macroinvertebrates along the altitude gradient

Five macroinvertebrate families, all showing dark bodies and cases, were morphologically identified at 3200 and 4000 m: mayflies (Ephemeroptera, Baetidae), caddisflies (Trichoptera, Limnephilidae), non-biting midges (Diptera, Chironomidae), blackflies (Diptera, Simuliidae) and flatworms (Turbellaria, Planariidae). At this taxonomic level, melanin concentration was twice as high at 4000 m (55.4–77.9 µg mg/dw) as at 3200 m (22.2–41.6 µg mg/dw), but did not differ among the five taxa nor between seasons (Figure 2).

Because we wanted to explore whether altitude-related differences in melanin concentration were due to changes in community composition or to population differentiation in dominant invertebrate species, five genera belonging to the mayfly family Baetidae occurring between 650 and 4000 m were identified morphologically: *Americabaetis*, *Dactylobaetis*, *Tupiara*, *Baetodes* and *Thraulodes*. For the first three genera

Table 1. Physicochemical variables at the five sampling sites in the Quilcayhuanca River and the Rímac River area, Peru.

Catchment	Season	Altitude (m)	UV-B (W/m^2)	Conductivity (µS/cm)	pH	Temperature (°C)	Oxygen (mg/L)	DOC (mg/L)	Depth (cm)	Discharge (L/s)	Velocity (cm/s)
Quilcayhuanca	Dry	3200	2.23	73	6.5	13.7	5.2	0.99	89	410	41
		4000	4.89	70	7.4	12.4	5.6	0.52	56	350	48
	Rainy	3200	1.08	29	7.3	10.0	5.9	1.26	101	640	62
		4000	2.47	52	7.5	9.7	6.0	0.73	70	560	73
Rímac	Dry	650	0.84	479	8.4	16.3	5.2	3.35	99	600	67
		1900	1.21	349	8.3	14.5	5.6	2.11	82	520	54
		3200	2.35	254	8.5	12.2	5.9	1.21	73	460	44
	Rainy	650	0.49	313	8.0	15.1	5.5	4.05	117	810	91
		1900	0.77	277	7.8	12.2	5.8	2.97	106	730	74
		3200	1.33	168	7.9	11.0	6.1	1.82	98	650	68

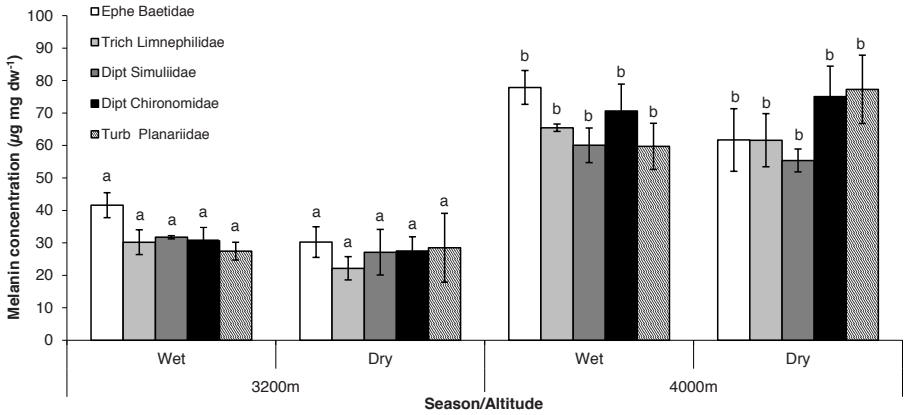

Figure 2. Melanin concentrations in benthic macroinvertebrate families during the wet and dry season at 3000 and 4000 m in the Quilcayhuanca River. Letters denote significant differences between altitudes

sufficient samples were available to identify them genetically. A blast search of all sequences returned similar sequences of mayflies, but no perfect match was found. The latter is not surprising, because mayfly sequences in Genbank originate from studies of North American species, while South American species are lacking. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+*G*, parameter = 1.4681)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 63.2768% sites). Codon positions included were 1st+2nd+3rd+Noncoding. The final dataset for the phylogenetic analysis contained 33 nucleotide sequences of 531 sites after alignment and trimming. There were in total 192 parsimony informative sites and no gaps in the dataset. Specimens within genera were divided into monophyletic groups, according to the altitude of the catchment site for *Americabaetis* and *Tupiara*, but not for *Dactylobaetis* (Figure 3). The monophyletic groups were likely to represent different species. The phylogenetic position of *Americabaetis* individuals at 1900 m could not be established due to failed DNA extraction.

Genetic species identification allowed melanin analysis per taxon per site. Since melanin concentration in mayflies at the generic level (data not shown) did not differ significantly between seasons, we present only data from the dry season. In all five mayfly genera, melanin concentration increased with altitude (Figure 4). In *Americabaetis* melanin increased significantly ($P < 0.02$) from 1900 to 3200 and 4000 m, increasing five-fold with increasing altitude. In *Dactylobaetis* and *Tupiara* melanin concentrations were almost twice as high at 1900 m as at 650 m ($P < 0.02$). Individuals belonging to the morphologically identified genus *Baetodes* contained similar melanin concentrations at 650 and 1900 m ($P >$

0.05), which were more than doubled at 4000 m, while *Thraulodes* showed almost a three-times higher melanin concentrations at 4000 as at 3200 m ($P < 0.05$).

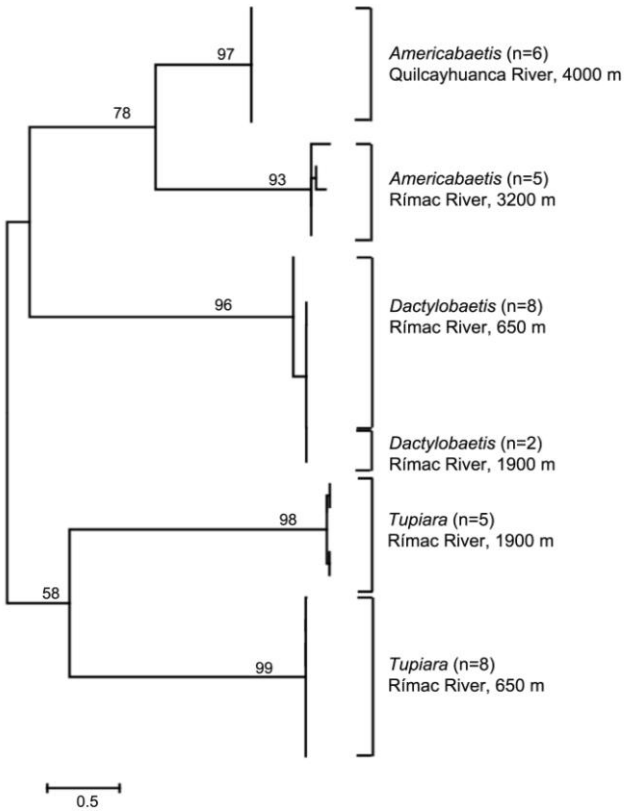

Figure 3. Phylogenetic relationships of Baetidae taxa from the Quilcayhuanca River and the Rímac River catchments. The best fitting substitution model used for inferring the maximum likelihood tree was the HKY+G+I model (G = 1.4681, and I = 0.6328). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown above the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *n* indicates the number of individuals analyzed per taxon.

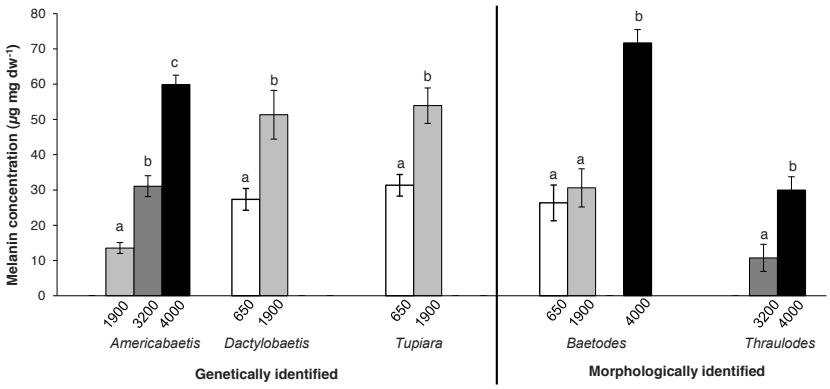

Figure 4. Melanin concentrations in mayfly taxa identified genetically and morphologically along an altitude gradient. For *Americabaetis*, *Dactylobaetis* and *Tupiara* genera, taxonomic clades corresponding to different altitudes are shown, excepting *Americabaetis* at 1900 m which could only be identified morphologically. Letters denote significant differences between altitudes.

Discussion

Andean streams share common aspects with highland streams from other mountainous areas, such as low water temperature, low oxygen (because of the low atmospheric pressure) and nutrient concentrations, but their unique feature is the very intense solar irradiance due to the thinner ozone layer at low latitudes and proximity to the equator. Hence, the UV-B values measured in the present study in the high Andes are among the highest irradiances reaching the Earth's surface. Given the steep UV-B gradient of 0.49 to 4.89 W/m^2 in the Andes, we hypothesized that differences in pigment concentration would be a constitutive trait or adjustable response of macroinvertebrates species depending on their altitudinal range. Indeed, our study is among the first to describe high melanin pigmentation in benthic macroinvertebrates from high altitude streams (>3500 m). The highest melanin concentrations, in Baetodes (71.7 µg mg/dw) and Americabaetis (59.8 µg mg/dw) from the highest sampling sites, were similar only to those measured in the cladocerans Daphnia himalaya (85 µg mg/dw) from Himalayan lakes (Sommaruga, 2010), and Scapholeberis mucronata (68.4 µg mg/dw) from subarctic and arctic ponds in northern Canada and Alaska (Rautio et al., 2009). These values were far above other melanin concentrations measured in alpine and arctic Daphnia species (0.03 µg mg/dw, Hansson et al., 2007; 0.037 µg mg/dw, Hobaek and Wolf, 1991; 0.31 µg mg/dw; Hebert and Emery, 1990; 4.7 µg mg/dw, Connelly et al., 2009; Rautio et al., 2009; 30 µg mg/dw,

Rautio and Korhola, 2002), and in the fairy shrimps Branchinecta paludosa (4.4 µg mg/dw, Rautio et al., 2009) and Artemiopsis stefanssoni (2.3 µg mg/dw, Rautio et al., 2009).

At our study sites, the strong effect of the UV-B gradient on melanin concentrations in macroinvertebrates was probably accentuated by the coinciding decreasing levels of DOC with altitude. Also, under the increased UV-B conditions in the high Andes, DOC may have become photobleached, degrading the chromophores that absorb light and significantly lowering its capacity to attenuate the penetration of UV-B in the water column (Zepp et al., 2007). Hence, the low concentrations of DOC in high altitude streams, and its eventual photodegradation, may have left benthic communities in shallow streams more exposed to elevated UV-B, thus relying even more on melanization to avoid the negative effects of solar radiation at the highest altitude sites. Moreover, the low temperatures of high altitude Andean streams inhibit the enzymatic repairing of UV-B damage to proteins and nucleic acids (Roos and Vincent, 1998). Although this has been described in cyanobacteria, it could have also exacerbated the impacts of UV radiation on benthic macroinvertebrates through similar mechanisms.

The persistence of species under high solar radiation may be based on avoidance behaviour related to preferences for habitats providing physical refuge from the UV-B radiation at high altitude sites, such as aquatic vegetation, stones and the sediment, or on opaque cases for caddisflies. However, our results suggest that the melanin concentration in macroinvertebrates, which was strongly correlated with increasing UV-B between 650 and 4000 m, may well represent a major defence against the damaging effects of intense solar radiation in the Andes, allowing these macroinvertebrates to persist under such harsh conditions. Moreover, the melanin concentrations did not show any significant difference between the wet and dry season at specific altitudes, suggesting that it may be a constitutive response to the high UV-B throughout the year in the high Andes, despite the costs of synthesis. These costs may be associated with dietary limitations on melanin precursors, and energy allocation for melanin production in the exoskeleton, including its re-synthesis after each moult. In addition to dark pigmentation to protect against UV damage, melanin has other functions, such as cuticle hardening, which may result in competing demands on melanin precursors, resulting in trade-offs unique to some taxa (Stoehr, 2006; Sugumaran, 2002). The presently observed constitutive melanin production contrast with the strong seasonal patterns in melanin contents described in subarctic *Daphnia*, which synthesized pigments only during the open-water summer months, starting after the ice cover period (Rautio and Korhola, 2002).

To determine if the observed differences in pigment concentration between individuals from different sites and seasons resulted from the selection of species making up the local community or from the intraspecific differentiation of populations, macroinvertebrates were identified to species using genetic sequencing. In *Tupiara* and

Americabaetis, different altitudes were inhabited by different species. In *Tupiara*, two species were found in the Rímac River, but spatially separated by altitude, indicating that despite the potential for dispersal of adult mayflies, larval drift and subsequent gene flow along the same catchment, each species had different habitat preferences, resulting in a restricted, non-overlaping species distribution pattern. Although this may explain why the species from 650 m were not found at 1900 m, it does not explain the reverse, especially not in the same river. It is possible that melanin production confers a fitness cost at lower altitudes and, in consequence, high altitude species might be outcompeted by low altitude species. A similar pattern was found in *Americabaetis*, although we can not rule out the possibility that species distribution was influenced by the fact that sampling sites were disconnected and in two distant mountain ranges, at 4000 m from the Cordillera Blanca and at 3200 m from the Rímac River valley.

In contrast to *Tupiara* and *Americabaetis*, a single species of *Dactylobaetis* was present at two different altitudes within a single catchment, which suggests that melanin in this species varies according to UV exposure and that it can survive over a wide altitudinal range, thus probably representing a case of phenotypic plasticity, as proposed for Arctic copepods (Hessen et al., 1999; Hansson, 2004). Solar UV radiation induces defences in aquatic invertebrates including behavioural responses, such as vertical migration (Rhode et al., 2001), and phenotypic responses, such as the accumulation of photoprotective pigments (Hessen et al., 2002). Since in our shallow fast flowing streams, avoidance behaviour would be of limited benefit because of a little aquatic vegetation and sparse stone refuges, melanization may be the only viable phenotypic response regardless of high physiological costs (Gerrish and Cáceres, 2003).

In conclusion, phylogenetic analysis enabled us to demostrate two different patterns in mayfly species distribution associated with the altitudinal gradient. One pattern, based on adjustable (intraspecific) quantities of melanin, conformed observations in various invertebrate species made under moderate UV-B exposure. The second pattern, based on constitutive, high melanin concentrations, may be restricted to the high altitude invertebrate fauna thriving under the extreme UV-B regime of mountain ranges in the tropics.

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