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

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Raúl Augusto Loayza-Muro

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Faculteit der Natuurwetenschappen, Wiskunde en Informatica

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Raúl Augusto Loayza-Muro
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“What is written without effort is in general read without pleasure”
Samuel Johnson

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Chapter 1

General Introduction
Mountain regions cover about 27% of the Earth’s surface, and are dominated by the Andes, the Rocky Mountains, the Himalayas, the Alps and the Atlas ranges (Figure 1). These regions encompass glacial stocks and freshwater reservoirs that play a valuable role providing ecosystem services to large human populations living downstream (e.g. drinking water, food, and electric power, or purifying water). Mountain regions are unique through the heterogeneity of ecosystems and diversity of climates arising from their sharp altitude gradients. When approaching the summits, these factors create exceptional harsh conditions for life, which may be challenged even further by man-made disturbances: life at the edge.

![Profile of the latitudinal position of altitude belts in mountains across the globe. Grey is montane; black is alpine; white is the nival belt (Körner, 2003).](image)

**Life in high altitude streams**

Alpine streams are perhaps our most important water sources, but are at the same time one of the least studied ecosystems in the world. They are located above the tree line, which ranges in elevation from near sea level at high latitudes, to almost 4000 m above sea level (a.s.l.) in tropical mountains. High altitude streams show particular environmental characteristics, such as banks consisting of bedrock or mineral sediments, devoid of dense riparian vegetation; little wood debris and a low organic matter content due to the presence of only herbaceous plants and small shrubs; and a limited autotrophic production governed by low temperatures and nutrient levels. With increasing altitude environmental conditions,
such as water temperatures, oxygen levels and nutrient concentrations become more extreme and consequently, the diversity of aquatic communities in mountain streams exhibits a decline towards the summits (Vinebrooke and Leavitt, 1999; Miserendino, 2001; Jacobsen, 2004; Rostgaard and Jacobsen, 2005; Jacobsen and Marin, 2007; Jacobsen 2008a). Under these extreme conditions, in invertebrates such as mites, collembolans, dipterans, coleopterans, ephemeropterans and trichopterans, an array of selected traits including small body size, clinger habits, short life cycles, photoprotective pigmentation, sediment dwelling and case-protection confer different adaptive strategies to cope with the stressful habitats typical of high altitude streams (Townsend and Hildrew, 1994; Snook and Milner, 2002).

Altitude governs several environmental parameters known to be crucial for biota. Temperature regimes tend to be extreme at high altitude and indirectly affect water discharge rates in streams. Low air pressure diminishes the oxygen concentrations in water, while more ultraviolet (UV) radiation penetrates the thinner atmosphere above high mountains. These correlated factors act together on alpine biota and the integrated effect may be more important than the effects of the single factors. Accordingly, vertical zonations of species distribution and variations in community structure have been better explained by altitude than by small-scale factors associated with the specific habitat (Jacobsen, 2003; Finn and Poff 2005). Yet, life at the highest altitudes (> 4000 m) has seldomly been studied. Because of the unique suite of environmental conditions at such heights, the present project selected high altitude as potential driver of the composition of benthic invertebrate assemblages.

A blistering solar radiation

The Tropical Andes is the only large mountain range on the equator, covering approximately 1 542 644 km² across Venezuela, Colombia, Ecuador, Peru and Bolivia (Figure 1). Andean streams exhibit all the aspects of high altitude streams discussed above, like low water temperatures, oxygen levels and nutrient concentrations, but the unique striking feature is the very intense solar irradiance due to the thinner ozone layer over low latitudes and its proximity to the equator. Above 4000 m, irradiance is up to 50% higher than that at sea level for an equivalent atmospheric moisture regime (Jacobsen, 2008b; Sevink, 2009), the highest levels being recorded between May and August, coinciding with a reduced cloudiness and rainfall. These values (4.2 W/m², Zaratti, 2003) stand out exceeding those registered in temperate and high latitude alpine areas (0.1 W/m², Cabrera et al., 1997; 0.17 W/m², Vinebrooke and Leavitt, 1999; 0.5 W/m², Kiffney et al., 1997b; 1.6 W/m², Kelly et al., 2003), and are among the highest irradiances reaching the Earth’s crust.
Solar radiation, especially UV-B radiation (280–320 nm), may shape the structure and function of aquatic communities by inhibiting primary production (Kinzie et al., 1998), altering the abundance and richness (Kiffney et al., 1997a), and the distribution of sensitive species, influencing trophic interactions (Kelly et al., 2003) and inducing DNA damage (Macfadyen et al., 2004). In high altitude Andean streams, macrobenthic communities are constantly exposed to blistering levels of UV-B, further accentuated by the lack of shaded areas and dissolved organic matter, which are two major factors attenuating the penetration of solar radiation (Cabrera et al., 1997; Clements et al., 2008). This exposure may become most intense during dry season low-water flow conditions, when radiation levels reaching the Earth's surface peak (Kiffney et al., 1997a). Thus macrofauna thriving in high altitude Andean streams must be especially adapted to counteract the potential adverse effects of the highest UV-B radiation levels on Earth.

To cope with the effects of UV-B radiation, aquatic organisms commonly acquire or synthesize photoprotective compounds that function either as sunscreens or as scavengers of photo-produced reactive oxygen species (ROS; Vinebrooke and Leavitt, 1999). Among copepods, such pigments are carotenoids and mycosporine-like amino acids (MAAs; Sommaruga and García-Pichel, 1999; Hansson, 2000). For cladocerans, such as *Daphnia*, and isopods with a thick chitin exoskeleton, melanin is the most important pigment, and highly melanized specimens are almost exclusively found in arctic or high altitude environments (Hebert and Emery, 1990; Sommaruga, 2010). Several studies have shown that melanized organisms can tolerate high solar irradiances better than non-pigmented relatives, and that the level of pigmentation is an inducible and adjustable defense mechanism (Hessen et al., 1999; Rautio and Korhola, 2002; Hansson, 2004, Hansson et al., 2007). Hence, it is expected that macroinvertebrates inhabiting high altitude Andean streams under exceptional high solar radiation levels should show a strong expression of UV protection mechanisms, enabling specialized taxa to persist on the ‘UV boundaries of life’.

**Natural and anthropogenic metal input**

Metals are natural, ubiquitous but unevenly distributed constituents of the Earth’s crust. In mountain areas like the Andes, metals are readily mobilized by acid conditions produced by natural oxidation of mineral layers. The tropical Andes, in particular the Cordillera Blanca in Peru, show a unique and diverse geomorphology consisting of a granodioritic batholith formed by plagioclase and biotite minerals presenting aluminum, iron, nickel, cobalt, strontium and zinc (Rivera et al., 2008; Sevink, 2009). At the upper sections in the proglacial zone of the Cordillera Blanca, metamorphic sedimentary rocks characterized by pyrite are well oxidized, generating protons that lower the pH below 4. As
a result, rocks are readily weathered resulting in high metal concentrations being mobilized into streams (Burns, 2010). Thus, the heterogenous morphology results in a prominent spatial diversity in leaching along the Andes. However, the most important contributor to metal pollution of rivers is mining, encompassing abandoned mines, acid-mine drainage and mine tailings releasing high concentrations of metal residues into river systems. As a consequence, metals are still one of the most ubiquitous and persistent sources of environmental contamination.

Benthic macroinvertebrates are directly or indirectly impacted by metals in the water (Kiffney and Clements, 1996b), substratum and food resources (Kiffney and Clements, 1993; Farag et al., 1998; Courtney and Clements, 2002), and show different responses to metal exposure. Metals may therefore explain much of the variability in assemblage structure between polluted and unpolluted sites, and reveal species-specific sensitivities to metals (Clements et al., 2000). Studies on the effects of metals in natural and artificial polluted streams have shown a loss of sensitive species, resulting in a significant reduction of richness and a shift towards more tolerant taxa (Gerhardt et al., 2004). Ephemeroptera are among the most sensitive group, whereas Plecoptera, Trichoptera and Diptera may survive under high metal concentrations (Clements, 1994; Kiffney and Clements, 1994; Clements et al., 2000). Indirect effects of metal pollution include smothering of the streambed by metal oxyhydroxide precipitates, restricting available habitats for benthic fauna, impoverishing food quality, and modifying interactions between functional feeding groups (Kiffney, 1996; Clements, 1999; O’Halloran et al., 2008).

To cope with metal toxicity, aquatic invertebrates display different physiological mechanisms for detoxifying or regulating internal metal concentrations, like the expression of metal-binding metallothioneins and melanin (McGraw, 2003; Timmermans et al., 2005), changing the ion permeability of cuticle or gill epithelium (Hare, 1992), and shedding of metals during molting (Timmermans and Walker, 1989). Genetic adaptation has also been considered as a mechanism of metal tolerance in polluted environments (Groenendijk et al. 2002; van Straalen et al. 2005; Buchwalter et al. 2008), suggesting that metals can act as a selective factor structuring aquatic communities. This triggered us to study metals as a potential driving force in benthic invertebrate community composition and to answer the question if selection for stress tolerance in organisms thriving under extreme metal conditions is due to a remarkable adaptive capacity of few species or to a large diversity of species with different capacities to cope with extreme environments.

**Taxonomic status of high altitude fauna**

Investigations on the effects of high altitude conditions on the invertebrate fauna of the tropical Andes require that this fauna has been taxonomically well identified, but this
is, however, not the case. Current taxonomic keys for the Andes (Roldán, 1996; Domínguez and Fernández, 2009) have a very low resolution and the identification of individual species from remote and high altitude communities is therefore often problematic and unreliable. However, this challenge is inevitable in a study on the fauna ‘at the edge of life’. Therefore, dedicated approaches are needed and the advance brought about by genome based taxonomy invites also genomic approaches to the high Andes macrofauna. So far genomic analysis of high alpine flora have been published (Manel et al., 2012; Xavier Pico, 2012), but equivalent studies on Andean macrofauna are non existing (Scheihing et al., 2011). The present day Andean fauna has been formed through regional diversification events driven by strong selection and local speciation (Hoorn, 2010). A genomic analysis of high altitude fauna in the Andes is therefore likely to reveal unique and new taxa that are difficult to link to classical morphologically identified taxa.

**Aim and objectives**

The aim of this thesis was to identify potential drivers of diversity in poorly studied benthic invertebrate assemblages in high altitude Andean streams and to elucidate the mechanisms that enable them to cope with ‘life at the edge’. The unresolved taxonomic status of high altitude fauna required the use of genetic analysis to amend morphological identification. To this purpose, the following objectives have been set:

- To describe for the first time the benthic invertebrate assemblages in high altitude Andean streams and to relate their composition to the strong gradients in abiotic factors.
- To unravel the role of melanin as a strategy against harmful UV-B radiation and metal exposure.
- To study the genetic diversity of benthic invertebrates occurring under extreme conditions and showing specific defense strategies.

**The tropical Andes: life at the edge**

The Cordillera Blanca in the Peruvian Andes contains the highest permanently flowing streams of glacial origin (4000–5000 m a.s.l.) and ~70% of all tropical glaciers (Vuille et al., 2008). However, these are particularly sensitive to, and the most visible indicator of global climate changes, since variations in naturally high solar regimes and rising atmospheric temperatures are accelerating their melting. Simultaneously, the retreat of ice masses is accelerating the weathering of metal-rich rocks, which produces natural acid drainage and mobilizes toxic metals into lagoons and streams, deteriorating the quality
of headwaters and aquatic biota (Ministerio de Energía y Minas, 1998; Sevink, 2009). These inputs, together with those originating from abandoned mine wastes and present mining, may create extreme detrimental conditions compared to those reported in temperate and other alpine streams (Kiffney and Clements, 1996a; Clements, 1999; Courtney and Clements, 2000; Clements et al., 2000).

To meet the objectives of the present study we selected streams distributed along an altitude range (650–4200 m a.s.l.) in the Peruvian Andes, encompassing a strong gradient of abiotic factors (Figure 2). We chose reference and metal-rich streams at each altitude, showing strong differences in water quality (e.g., pH, conductivity, transparency) and habitat conditions (e.g., riverine vegetation, streambed structure, water flow).

Figure 2. Vegetation distribution in Peruvian Andes, showing the position of reference and metal-polluted streams and the solar radiation along the altitudinal gradient. (Adapted from http://wahlclassroom.blogspot.com/2012/12/the-four-zones-of-andes-mountains.html).

Because of the steep Andean slopes, the streams selected in this study were in general fast-flowing and turbulent, particularly during the rainy season. Above 4000 m streams were shallow, showing riverine herbaceous plants and small shrubs providing poorly shaded areas, while bigger shrubs and trees belonging to the Andean forest were more abundant downstream. Reference streams were characterized by transparent waters, with substrates
consisting of gravel, pebble, and stones in runs and riffles at high altitude, and larger rocks along stream banks at low altitudes. In contrast, metal polluted sites showed more turbid waters and streambeds smothered by crusts of metal precipitates (Figure 3).

Figure 3. Streams in the Cordillera Blanca and Cordillera Negra, in the Peruvian Andes. A, reference stream in the Tuco gorge (4120 m); B, metal polluted Santiago stream in the Aija gorge (4200 m); C, reference stream in the Quilcay sub-catchment (3070 m); D, polluted stream in the Aija gorge (3100 m). Photos by R. Loayza-Muro.

Outline of the thesis

The aim of this thesis was to identify potential drivers of diversity in poorly studied benthic invertebrate assemblages in high altitude Andean streams and to elucidate the mechanisms that enable them to cope with ‘life at the edge’. To meet the objectives of the present study a combined field and experimental approach was designed, meanwhile progressing from traditional taxonomy towards genetic identification.

We first explored the effect of metal pollution on benthic invertebrate community composition in Andean high altitude streams (chapter 2). Physical chemical variables and metal concentrations were measured in reference and polluted streams, and macrofauna was sampled. To determine how the sampling sites were structured by the abiotic variables
Principal Component Analysis was applied. The effects of the principal variation in physical chemical variables on the faunal assemblages was analysed by means of Canonical Correspondence Analysis. This allowed us also to identify metal sensitive and insensitive taxa.

Since the Andes represent a sharp altitude gradient the differential effects of metal pollution and altitude on benthic macroinvertebrate community composition were evaluated in chapter 3. In addition to general physical chemical parameters and metal concentrations, now also UV-B irradiance and DOC concentrations were measured. To explain the patterns in faunal composition and to identify taxa thriving under combined high metal and UV-B conditions Canonical Correspondence Analysis was applied.

Next, chapter 4 explored the adaptive responses enabling specialized macrofauna to survive under these combined high metal and UV-B conditions, hypothesizing that melanin counteracts both the adverse effects of solar radiation and of metals. Therefore, melanin was determined in chironomids from reference and metal polluted streams at 3000 and 4000 m altitude. 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).

Melanization in macroinvertebrates inhabiting high altitude Andean streams as an adaptive response to high UV-B radiation was further studied in chapter 5. To this purpose we measured UV-B radiation from 650 to 4000 m and compared body melanin concentrations from several benthic macroinvertebrate orders sampled at these altitudes. To evaluate if altitude-related differences in melanin concentration between taxa were due to a variable community composition or to population differentiation, DNA sequencing was performed. This way, five genera belonging to the mayfly family Baetidae were genetically identified, allowing comparisons of melanin concentrations at the species level.

Chironomids are among the few taxa thriving under the harshest environmental conditions, the highest metal concentrations and UV-B levels. To study if the presence of chironomids in metal polluted Andean high altitude streams is attributable to population differentiation or changed species composition, the genetic composition of chironomid communities from reference and metal polluted streams at 3000 and 4000 m was determined by mitochondrial cytochrome oxidase I (COI) gene sequencing and construction of a phylogenetic tree in chapter 6.

Finally, the concluding remarks in chapter 7 discuss the main findings of this thesis, focusing on the environmental factors that drive the diversity of benthic invertebrate assemblages in high altitude Andean streams, and on the mechanisms enabling them to cope with this harsh environment.
Chapter 2

Metal induced shifts in benthic macroinvertebrate community composition in Andean high altitude streams

Abstract

High altitude creates unique challenging conditions to biota that limit the diversity of benthic communities. Since environmental pollution may add further stress to life at high altitude, the present study explored the effect of metal pollution on macroinvertebrate community composition in Andean streams between 3500 to 4500 meters above sea level (m a.s.l) during wet and dry seasons. At polluted sites, showing a high conductivity and a low pH, metal concentrations (e.g. Al, 13.07 mg/L; As, 3.49 mg/L; Mn, 19.65 mg/L; Pb, 0.876 mg/L; Zn, 16.08 mg/L) ranged from 8 up to 3500 fold higher than at reference sites. The Cumulative Criterion Unit allowed quantifying the potential toxicity of metal mixtures at the contaminated sites. Principal Component Analysis of physical chemical variables showed that reference sites were more likely to be structured by transparency, water discharge and current velocity, while polluted sites appeared to be determined by metals and conductivity. Canonical Correspondence Analysis indicated a strong influence of highly correlated metals in structuring invertebrate communities, which were dominated by dipterans, coleopterans, collemobolans and mites at polluted sites. At reference sites crustaceans, ephemeropterans, plecopterans and trichopterans were the most representative taxa. It is concluded that severe metal pollution induced changes in macroinvertebrate community composition in high altitude Andean streams, with a replacement of sensitive taxa by more tolerant taxa. Yet, relatively species-rich communities persisted under harsh conditions.
Introduction

Andean high altitude streams are among the least studied freshwater ecosystems, although they represent unique challenging conditions, mainly due to low water temperature and low dissolved oxygen concentrations that limit the diversity of benthic communities (Jacobsen et al., 2003; Jacobsen and Marín, 2007; Jacobsen, 2008a). It is generally believed that cold high altitude streams are more oxygen rich than warm low land streams because of better oxygen solubility at lower temperatures and better aeration generated by fast-flowing, turbulent waters. This is not the case however, since oxygen pressure also decreases with altitude, rendering these streams close to a critical oxygen saturation level (Jacobsen, 2008b). Also the regime of UV exposure and temperature fluctuation tends to create challenging conditions for aquatic life (Jacobsen, 2008a; Tartarotti et al., 1999). Environmental pollution may add further stress to life at high altitude and these combined stressors may have a strong impact on local communities.

Mining exploitation has been one of the most important economic activities developed at high altitudes in Andean countries and is still growing. In the past, mining practices were performed without environmental protection and mineral waste was stored in large piles exposed to rainfall (Romero et al., 2008). Currently, these abandoned dumps and mine tailings represent a standing threat for Andean rivers and streams due to the continuous mobilization of metals and acid drainage, changing water chemistry and biotic communities (Ministerio de Energía y Minas, 1998). Moreover, since in several cases metal levels exceed the permissible limits for human or agricultural water use, it is deemed that such toxic contaminants have critically deteriorated important freshwater sources in the region over decades of exposure (UNEP, 2003).

Studies on the effects of increased acidity and dissolved metal concentrations (cadmium, copper, zinc) in natural and artificial streams in the Rocky Mountains (USA) have shown a significant reduction of invertebrate abundance and species richness, due to loss of sensitive taxa and a shift in community composition towards more tolerant species (Kiffney and Clements, 1994; Gerhardt et al., 2004). Indirect effects include smothering of the streambed substrate by metal precipitates, reducing the habitat availability for stream fauna, decreasing food quality, and modifying interactions between functional feeding groups (Clements, 1999; Courtney and Clements, 2002; O’Halloran et al., 2008). In addition, it has been reported that related species from elevated temperate streams (2500 m a.s.l.) are more sensitive to metals than those from low land streams (Kiffney and Clements, 1994; Kiffney and Clements, 1996). However, in spite of the complex geology of the Andes and the presence of an active mining industry, little attention has been devoted to the effects of metals and acid drainage on Andean high altitude streams. Hence, the aim of the present study was to determine if elevated metal concentrations represent a stress factor shaping benthic invertebrate community composition, comparing reference and metal polluted
streams at high altitude. To this purpose, benthic macroinvertebrate communities in reference waters and those exposed to natural and anthropogenic metal contamination were sampled during four consecutive seasons in 2006 and 2008 at six high altitude sites (3500 to 4500 m a.s.l.) in the Cordillera Blanca and Cordillera Negra (Peruvian Andes). Multivariate analysis was used to identify those environmental factors that most strongly influenced biodiversity and composition of macroinvertebrate assemblages.

Materials and methods

Study sites

In Central-Northern Peru (Ancash region), the Cordillera de los Andes comprise two parallel mountain ranges, the eastern Cordillera Blanca and the western Cordillera Negra, both extending beyond 5500 m a.s.l along the Santa River. Below the permanent snow-line, between 3700 to 4200 m a.s.l, slopes have been modified for small agriculture and cattle rearing, and also mining activities take place. Streams in this area are fast flowing, with substrate consisting of gravel, pebble and cobbles in runs and riffles. They show a very sparse macrophyte growth and are almost completely unshaded.

Six sampling sites were selected, all located between 3500 to 4500 m a.s.l. (Figure 1). Four reference sites were selected. Three reference sites (Honda, Aquilpo and Ishinca) were located in the Cordillera Blanca at 3500 m a.s.l. in three different streams and one reference site (Pacilla) was in the Cordillera Negra at 3800 m a.s.l. Two polluted sites were selected, both in the Santiago stream in the Cordillera Negra. The first one was located at 4500 m a.s.l, where the geological formations contain high concentrations of metals which cause the water to be polluted, even though there were no mining activities upstream of this site (Santiago natural pollution) (http://intranet2.minem.gob.pe/web/archivos/dgm/publicaciones/public03/mapas/12.jpg). The second polluted site was located at 3800 m a.s.l, downstream abandoned mines (Santiago mine pollution). All sites were sampled in March, June, September and December. The Santiago natural pollution site was not sampled in September because it became dry.

Physical chemical characteristics

Measurements of pH, temperature (°C), conductivity and dissolved oxygen were performed at each sampling site using a multi-parameter instrument equipped with SenTix® 41-3, TetraCon® 325-3 and CellOx® 325-3 probes (WTW Multi 340i, Weilheim, Germany). Transparency was measured with a 120-cm polycarbonate turbidity tube (Wildlife Supply Company, Buffalo, NY, USA). Stream depth was calculated from four measurements at each of three parallel cross-sections with a calibrated stick. Mean current velocity was obtained by timing a float three times as it moved over a distance of 10 m.
Discharge was calculated as the average of the three products of mean current velocity, mean depth and stream width at three cross-sections (Miserendino and Pizzolón, 2003). For determining hardness, phosphates and ammonia, water samples were taken below the water surface, kept at 4°C in a Styrofoam box, and analyzed using standard methods (Clesceri et al., 1998). Water samples for total metals were preserved in 10 N HNO₃ and analyzed by ICP-ES (induced-coupled plasma emission spectroscopy) (USEPA, 1994). Samples for determining hardness, phosphates, ammonia and metals were taken in triplicate at each sampling time.
Since the polluted sites were expected to contain a mixture of metals with potential additive effects, the cumulative criterion unit (CCU) (Clements et al., 2000) was calculated. The CCU is defined as the ratio between the stream metal concentration and the U.S. Environmental Protection Agency (U.S. EPA) criterion value for toxicity, summing the ratios for all metals measured at a specific site (USEPA, 1986): \[\text{CCU} = \sum \frac{m_i}{c_i},\] where \(m_i\) is the total recoverable metal concentration and \(c_i\) is the criterion value for the \(i\)th metal. The criterion value is based on U.S. EPA guidelines on critical concentrations, which may harm aquatic organisms when exceeded. CCU values are scaled as follows: < 1.0, no adverse effects; 1.0 to 2.0, adverse effects; 2.0 to 10.0, significant mortality to sensitive species and altered benthic community composition are expected; > 10.0, highest toxicity (Clements et al., 2000). Because water hardness affects the toxicity and bioavailability of some metals, criterion values for Cd, Cu, Pb and Zn were modified to account for variation in water hardness between streams. For Al, Fe and Mn no adjustment was needed and we followed the U.S. EPA criterion values (USEPA, 1986).

**Invertebrate sampling**

At each sampling site, three Surber samples (each 20 cm\(^2\), mesh size 250 μm) were collected randomly from gravel-pebble substratum. In addition, a qualitative sample was collected for about 20 to 30 min, including all other possible microhabitats over representative sections, such as stones and stagnant water along the banks, using forceps and a white plastic tray. All samples were preserved in 70% (v/v) ethanol, and sorted in the laboratory with the use of a stereomicroscope Zeiss Stemi DV4 (Göttingen, Germany). Insects were identified to family level and most non-insects to order or class, using taxonomical keys (Merrit and Cummins, 1996; Roldán, 1996). The relative abundance of Ephemeroptera, Plecoptera and Trichoptera (% EPT) was calculated, because these groups are generally considered to contain species sensitive to environmental pollution.

**Statistical analysis**

A principal components analysis (PCA) based on a correlation matrix was used to describe the main variation in physical chemical variables between all samples. Prior to this analysis, environmental variables were checked for normality, and those not meeting a normal distribution, were log transformed. All variables were transformed except temperature, dissolved oxygen, current velocity, water discharge, and potassium concentration. Since the PCA revealed that most samples from the same site could not be considered as independent, the seasonal samples for each environmental variable were averaged. These averages were used in a one-way analysis of variance (ANOVA) to test for differences between the reference and polluted sites. Analyses were done in SPSS 16.0 (SPSS Inc., Chicago, IL, USA).
Canonical correspondence analysis (CCA) (ter Braak, 1986) was done to examine the effects of the principal variation in physical chemical variables (as extracted by the PCA, see above) on the faunal assemblage of all samples. In the CCA, we focused the scaling on so-called inter-species distances and applied Hill's scaling type (ter Braak, 1986). Prior to the CCA, the family abundances were log transformed. The significance of the first canonical axis and the two canonical axes together was assessed with a permutation tests using 499 permutations under a reduced model (ter Braak and Šmilauer, 2002). This analysis was done with CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA).

**Results**

**Physical chemical differences between reference and polluted sites**

Mean conductivity was lower and mean pH was higher in the reference streams compared to the polluted streams (Table 1). Except for Co, Fe, K, Mg, and Na, the mean concentrations of all metals were higher in the polluted streams than in the references streams (Table 2). The mean metal concentrations at the polluted sites (e.g. Al, 13.07 mg/L; As, 3.49 mg/L; Cd, 0.5 mg/L; Mn, 19.65 mg/L; Pb, 0.876 mg/L; Zn, 16.08 mg/L) ranged from 8 (Sr) to 3500 (As) times those at the reference sites, indicating a high degree of contamination with metals. The mean CCU ranged from 1.37 to 239.38, being significantly higher at the polluted sites. Although the CCU at reference sites exceeded 1.0, indicating metal pollution, the large and significant differences with CCU values at polluted sites allowed separating the two categories.

The loadings along the first PCA axis indicated that the principal variation in physical chemical variables was based on a positive high correlation between all metals and conductivity (Figure 2). These variables were, in turn, highly negatively correlated with pH, transparency, and, to a lower degree with current velocity, water discharge, and phosphates. As such, the first PCA axis clearly arranged the samples along a gradient of contamination: to the right the polluted sites and to the left the reference sites (Figure 2). The second PCA axis was positively loaded by phosphates, stream velocity, water discharge, Co and K, and negatively by N-ammonium and dissolved oxygen. Because most samples from each
Table 1. Means (± SD; n samples per site) for physical chemical variables at six sites in the Cordillera Blanca (*) and Cordillera Negra (+) area, Peru. $F$ and $p$ denote the results of the analysis of variance (ANOVA) test between the mean of the four reference sites and the mean of the two polluted sites. PO$_4$-P, phosphates; NH$_4$-N, ammonium nitrogen.

<table>
<thead>
<tr>
<th>Site group</th>
<th>n</th>
<th>Name</th>
<th>Conductivity (μS/cm)</th>
<th>Hardness (mg CaCO$_3$/L)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>PO$_4$-P (mg P/L)</th>
<th>NH$_4$-N (mg N/L)</th>
<th>Oxygen (mg/L)</th>
<th>Transparency (cm)</th>
<th>Velocity (cm/s)</th>
<th>Discharge (L/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>reference 4</td>
<td>Honda (*)</td>
<td>137.8 (32.9)</td>
<td>105.1 (29.3)</td>
<td>7.4 (0.4)</td>
<td>10.8 (2.3)</td>
<td>0.64 (0.02)</td>
<td>0.023 (0.016)</td>
<td>5.08 (0.64)</td>
<td>100 (0)</td>
<td>63.0 (15.0)</td>
<td>867.5 (291.4)</td>
<td></td>
</tr>
<tr>
<td>reference 4</td>
<td>Aquilpo (*)</td>
<td>45.0 (12.7)</td>
<td>85.5 (21.5)</td>
<td>7.6 (0.4)</td>
<td>11.6 (1.5)</td>
<td>0.53 (0.06)</td>
<td>0.020 (0.011)</td>
<td>5.13 (0.48)</td>
<td>120 (0)</td>
<td>52.8 (13.3)</td>
<td>748.8 (263.1)</td>
<td></td>
</tr>
<tr>
<td>reference 4</td>
<td>Ishinca (*)</td>
<td>45.8 (7.7)</td>
<td>102.3 (13.3)</td>
<td>7.7 (0.4)</td>
<td>10.9 (1.6)</td>
<td>0.63 (0.10)</td>
<td>0.030 (0.024)</td>
<td>5.16 (0.76)</td>
<td>120 (0)</td>
<td>60.3 (14.8)</td>
<td>680.0 (236.2)</td>
<td></td>
</tr>
<tr>
<td>reference 4</td>
<td>Paclla (+)</td>
<td>118.9 (51.5)</td>
<td>95.0 (26.7)</td>
<td>7.2 (0.4)</td>
<td>12.8 (1.4)</td>
<td>0.50 (0.10)</td>
<td>0.045 (0.070)</td>
<td>5.18 (0.95)</td>
<td>120 (0)</td>
<td>31.5 (10.3)</td>
<td>125.0 (86.9)</td>
<td></td>
</tr>
<tr>
<td>polluted 3</td>
<td>Santiago natural pollution (+)</td>
<td>410.2 (292.8)</td>
<td>130.0 (45.6)</td>
<td>4.2 (0.2)</td>
<td>9.3 (0.6)</td>
<td>0.50 (0)</td>
<td>0.057 (0.081)</td>
<td>5.62 (1.22)</td>
<td>120 (0)</td>
<td>9.3 (5.5)</td>
<td>20.3 (9.1)</td>
<td></td>
</tr>
<tr>
<td>polluted 4</td>
<td>Santiago mine pollution (+)</td>
<td>1776.0 (760)</td>
<td>1026.5 (377.8)</td>
<td>3.4 (0.2)</td>
<td>11.5 (1.3)</td>
<td>0.50 (0)</td>
<td>0.025 (0.030)</td>
<td>5.00 (0.74)</td>
<td>35 (44)</td>
<td>29.0 (10.4)</td>
<td>152.5 (70.4)</td>
<td></td>
</tr>
</tbody>
</table>

$F$ and $p$ denote the results of the analysis of variance (ANOVA) test between the mean of the four reference sites and the mean of the two polluted sites.
Macroinvertebrates in metal polluted streams

Table 2. Means (± SD; n samples per site) of metal concentrations and Cumulative Criterion Unit (CCU) at six sites in the Cordillera Blanca (*) and Cordillera Negra (+) area, Peru. $F$ and $p$ denote the results of the analysis of variance (ANOVA) test between the mean of the four reference sites and the mean of the two polluted sites. Highest/lowest metal concentration indicates the ratio of the highest to the lowest mean metal concentration.

| Site group | n  | Site       | Al    | As    | Ca    | Cd    | Co    | Cu    | Fe    | K     | Mg    | Mn    | Na    | Pb    | Sr    | Zn    | CCU  |
|------------|----|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|
| reference  | 4  | Honda (*)  | 0.92  | 0.009 | 11.15 | 0.001 | 0.028 | 0.003 | 1.50  | 0.97  | 3.15  | 0.36  | 1.95  | 0.010 | 0.06  | 0.12  | 10.77|
| reference  | 4  | Aquilpo (*)| 0.11  | 0.001 | 4.55  | 0.001 | 0.004 | 0.001 | 0.12  | 0.36  | 0.56  | 0.01  | 2.05  | 0.001 | 0.05  | 0.01  | 1.37  |
| reference  | 4  | Ishinca (*)| 0.59  | 0.001 | 5.43  | 0.001 | 0.003 | 0.001 | 0.45  | 0.59  | 0.63  | 0.03  | 1.50  | 0.001 | 0.06  | 0.02  | 4.95  |
| reference  | 4  | Paclla (+)  | 0.16  | 0.006 | 8.78  | 0.003 | 0.004 | 0.001 | 0.13  | 0.63  | 1.03  | 0.03  | 2.78  | 0.004 | 0.06  | 0.36  | 5.84  |
| polluted   | 3  | Santiago natural pollution (+) | 3.89  | 0.027 | 33.80 | 0.074 | 0.002 | 0.108 | 0.57  | 0.40  | 1.50  | 2.07  | 2.63  | 0.051 | 0.13  | 7.68  | 103.7 |
| polluted   | 4  | Santiago mine pollution (+)    | 13.07 | 3.490 | 59.76 | 0.501 | 0.092 | 1.011 | 37.48 | 1.33  | 17.13 | 19.65 | 5.88  | 0.876 | 0.39  | 16.08 | 239.38|
| Highest/lowest metal conc. |   |            | 118   | 3490  | 13    | 501   | 92    | 1011  | 312   | 4     | 31    | 1965  | 4     | 876   | 8     | 1608  | 33.5  |

$F$ and $p$ denote the results of the analysis of variance (ANOVA) test between the mean of the four reference sites and the mean of the two polluted sites. Highest/lowest metal concentration indicates the ratio of the highest to the lowest mean metal concentration.
stream were located near to each other in the ordination diagram, the PCA suggested that the physical chemical properties of the streams did not substantially differ between the seasons.

Figure 2. Principal components analysis (PCA) of physical chemical variables at six sites in the Cordillera Blanca and Cordillera Negra area in Peru. The arrows represent the loadings. Labels for stream are as follows: H = Honda, A = Aquilpo, I = Ishinca, P = Paclla, SN = Santiago natural pollution, SM = Santiago mine pollution; Numbers indicate month: 1 = March, 2 = June, 3 = September, 4 = December. Reference sites are represented by open symbols and polluted sites by solid symbols. PCA axis 1 explained 60%, and axis 2, 12% of the variance.

Relationship between physical-chemical water characteristics and community composition

A total of 45 families of aquatic insects and 10 other taxa were identified (Supplemental Data, Table S1). Among the insects, Diptera (15), Coleoptera (8), Trichoptera (7) and Collembola (7) were represented by the highest number of families. At the reference sites Honda, Aquilpo and Ishinca, Perlidae (Plecoptera) and Simuliidae (Diptera) had the highest number of individuals, while at both polluted sites in the Santiago stream chironomids were most abundant. Reference and polluted sites did not differ in mean abundance or family richness (Table 3) and the seasonal variation in faunal composition within streams was lower than the variation between streams (Figure 3A).
Table 3. Mean (± SD; n samples per site) abundance and family richness at six sites in the Cordillera Blanca (*) and Cordillera Negra (+) area, Peru. *F* and *p* denote the results of the analysis of variance (ANOVA) test between the mean of the four reference sites and the mean of the two polluted sites.

<table>
<thead>
<tr>
<th>Site group</th>
<th>n</th>
<th>Site</th>
<th>Abundance</th>
<th>Family richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>reference</td>
<td>4</td>
<td>Honda (*)</td>
<td>36.3 (10.0)</td>
<td>5</td>
</tr>
<tr>
<td>reference</td>
<td>4</td>
<td>Aquilpo (*)</td>
<td>93.8 (41.7)</td>
<td>13</td>
</tr>
<tr>
<td>reference</td>
<td>4</td>
<td>Ishinca (*)</td>
<td>74.8 (26.7)</td>
<td>14</td>
</tr>
<tr>
<td>polluted</td>
<td>3</td>
<td>Paclla (+)</td>
<td>1915 (1308)</td>
<td>36</td>
</tr>
<tr>
<td>polluted</td>
<td>4</td>
<td>Santiago natural pollution (+)</td>
<td>960 (1264)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>57.3 (70.1)</td>
<td>22</td>
</tr>
</tbody>
</table>

The differences in faunal assemblages between the three reference sites in the Cordillera Blanca and the reference site in the Cordillera Negra were mostly due to Chironomidae, Tabanidae (Diptera), Curculionidae, Dytiscidae, Elmidae and Scirtidae (Coleoptera), which were more abundant in the Paclla stream. In the Cordillera Negra, the faunal samples from the reference site Paclla stood out from the samples from the two polluted sites. Cladocerans, amphipods, ephemeropterans (Heptageniidae, Leptophlebiidae, Baetidae, Potamanthidae) plecopterans (Gripopterygidae, Perlidae) and trichopterans (Limnephilidae, Hydrobioscidae. Odontoceridae) dominated the reference sites and were completely absent from the polluted ones. The relative abundance of mayflies, stoneflies and caddisflies (% EPT) was higher at the reference sites, especially in the Aquilpo stream (32 to 67%), whereas dipterans (Ceratopogonidae, Chironomidae, Dixidae, Empididae, Tabanidae) and coleopterans (Dytiscidae, Gyrinidae, Hydrophilidae, Staphylinidae) were abundant at the contaminated sites (76 to 100%).

The CCA ordination biplot (Figures 3A and 3B) shows how the macroinvertebrate family composition depended on the principal variation in physical chemical variables, as represented by the first two PCA axes. The latter are shown as arrows which point in the direction of strongest influence on the main patterns in the faunal assemblages. The longer the arrows, the stronger the influence. The CCA analysis showed that the macroinvertebrate family composition at the polluted sites, especially downstream the mines, stood out because of their strong and positive correlation with the first PCA axis (the metal contamination factor). The optima of Ephydridae, Muscidae, Phoridae, Scatophagidae (Diptera), Actaletidae, Arrhopalitidae, Entomobryidae, Sensiphoridae (Collembola), Ptiliidae (Coleoptera), Anyphaenidae and Linyphiidae (Arachnida) were associated with high values of the first PCA axis (Figure 3B). In contrast, Baetidae, Heptageniidae, Leptophlebiidae
Figure 3. Canonical correspondence analysis (CCA) of faunal assemblages against the principal variation in physical environmental variables as extracted by PCA (see Figure 2), on the basis of 23 samples from six sites in the Cordillera Blanca and Cordillera Negra area in Peru. The eigenvalues of axis 1 and axis 2 were 0.61 and 0.46, respectively. Both axes explain 28% of the family data. Axis 1 and
both axes 1 and 2 together were significant at $p<0.01$. A: biplot of samples and explanatory variables. Sample labels correspond to the legend in Figure 2. Reference sites are represented by open symbols and polluted sites by solid symbols. B: biplot of families and explanatory variables. EPT taxa are identified as solid symbols. Family codes as in Supplemental Data Table S1.

(Ephemeroptera), Hydroptilidae, Limnephilidae (Trichoptera), Sarcophagidae (Diptera) and Amphipoda (Crustacea) seemed more sensitive to metal contamination, because these taxa only thrived in reference streams.

Discussion

The present study is among the first to describe macrofauna assemblages from high altitude streams in the Peruvian Andes. The major groups of benthic invertebrates, such as Ephemeroptera, Plecoptera, Trichoptera, Diptera and Coleoptera were well represented among the reference streams, with Baetidae, Perlidae, Limnephilidae and Chironomidae being the dominant families. The total number of taxa (55) is in accordance with or even higher than data on invertebrate assemblages from other Andean streams at similar altitude ranges in Ecuador (29 to 60) (Monaghan et al., 2000; Jacobsen, 2008b), Peru (40) (Acosta, 2009) and Bolivia (26) (Jacobsen and Marín, 2007). Since knowledge of Peruvian stream fauna and South American streams in general is scarce, the taxa could only be identified with certainty to the family level. This relatively coarse level of taxonomic resolution considers the high correlation between family richness of insects at individual stream sites and species richness (Bournaud et al. 1996), and may allow comparative analyses of community structure and detecting effects of pollution on benthic communities (Vanderklift et al., 1996).

At reference sites a strong relationship between community composition and water discharge was revealed by the canonical ordination. Indeed, the families Perlidae, Grippopterygidae (Plecoptera), Odontoceridae (Trichoptera) and Simuliidae (Diptera), had their highest abundance in streams with steep slopes and high water flow in the Cordillera Blanca. Such conditions are common in high altitude Andean streams, providing adequate conditions for development of these taxa. In contrast, the reference stream in the Cordillera Negra (Paclla) was dominated by dipterans and coleopterans preferring low water flow and discharge, and hence showed the highest abundance and richness towards the dry season. This observation agrees with several studies describing the influence of stream velocity on invertebrate communities (Miserendino and Pizzolon, 2003; Scheibler and Debandi, 2008). Although the second PCA axis used in the CCA also correlated with other factors such as phosphates and ammonia, these did not show a large variation between reference and
polluted sites and thus were not considered as having a relevant influence on community composition.

The results of the physical chemical analyses revealed unprecedented high metal concentrations at polluted sites. Low pH conditions have likely increased the bioavailability of metal ions and the turbidity of the water column, both having detrimental effects on aquatic organisms (Courtney and Clements, 2002). Simultaneously, the formation of stable orange precipitates and encrusted layers comprising iron oxyhydroxides has completely smothered the streambed, impoverishing food and substrate quality, and restricting available habitats for benthic fauna (Courtney and Clements, 2002; O’Halloran et al., 2008). The structuring role of metals was more evident during the dry season, as seen in other scenarios where increased metal levels and decreasing pH coincided with a decreased abundance of individuals and family richness (Gerhardt, 1993; Clements, 1994). The present study also demonstrated the importance of considering naturally occurring high metal concentrations, which had important consequences for community composition. Naturally and mine polluted sites shared 10 families, while presenting 9 and 11 site-specific families, respectively.

For some metals, their theoretical individual impact may be estimated from a species sensitivity distribution derived from individual median lethal concentration (LC50) values, revealing that the highest cadmium concentration in these streams would affect 50%, copper 95% and zinc 85% of the species (USEPA, 2005). However, because metal concentrations were highly correlated, an increased effect on community composition should likely be caused by all metals jointly. This was indicated by the high CCU values at the polluted sites (104 and 239), largely exceeding the upper cutoff of 10.0, which represents metal mixtures causing mortality and altering community structure (Clements et al., 2000). These results suggest that elevated metal levels play a relevant role in structuring benthic macroinvertebrate assemblages in Andean high altitude streams.

Surprisingly, however, the overall richness and abundance between reference and polluted sites did not differ significantly, which suggest that these metrics may not be adequate in assessing the effects of metal pollution on community composition when many species are being replaced. This was typically the case in the present study, where the high metal concentrations clearly induced a shift towards metal-tolerant families of dipterans, coleopterans and collembolans at contaminated sites, where dipterans (e.g. chironomidae) may probably represent the prey for predatory coleopterans. Regardless of metal origin, the present study showed that the diversity of macroinvertebrates was substantial at polluted sites, in spite of the challenging conditions associated with high altitude. We considered this richness to be high taking into account the extreme pollution and environmental conditions found here compared to similar studies (McNight and Feder, 1984; Winterbourn et al., 2000; Hirst et al., 2002; Gerhardt et al., 2004; Löhr et al., 2006).
The canonical ordination indicated that the abundance of specific taxa such as Ephydridae, Muscidae, Phoridae, Scatophagidae (Diptera), Actaletidae, Arrhopalitidae, Entomobrydae, Sensiphorura (Collembola), Ptiliidae (Coleoptera), Anyphaenidae and Linyphiidae (Arachnida) was higher along the first PCA component, which represents metal pollution, being determined by almost all highly correlated metals. In agreement it has been reported that Phoridae (Sorensen et al., 2009), collembolans (Crouau and Pinelli, 2008) and Linyphiidae (Jung et al., 2008) are able to develop and survive in metal polluted sites, suggesting that these taxa are less sensitive to the high concentrations of metals, low pH and altitude conditions encompassed by the present study. Although the drying of the naturally polluted site during the dry season could represent a confounding factor, the CCA indicated that the effect of metal pollution superseded any potential impacts related to this event.

Not all dipterans and coleopterans were able to thrive under high metal conditions, however. Sarcophagidae, Tabanidae (Diptera), Elmidae, Hydrophilidae and Scirtidae (Coleoptera) only appeared in the reference Paclla stream, suggesting that unpolluted tributaries may serve as refuge for sensitive invertebrate taxa and as potential sources of colonizers following restoration (Courtney and Clements, 2002). The polluted sites also excluded mayflies, stoneflies, caddisflies, amphipods and cladocerans, which only appeared in pristine waters. This trend was expected since the sensitivity of EPT taxa towards acid pH and metals has been well described in field surveys in low land acid mine areas and experimental microcosms (Gerhardt et al., 2004; O’Halloran et al., 2008; Peterson and van Eeckhaute, 1992). The sensitivity of EPT taxa was confirmed by the CCA, which clearly identified groups relatively sensitive to metal pollution, such as Baetidae, Heptageniidae (Ephemeroptera), Limnephilidae, Hydroptilidae (Trichoptera), Sarcophagidae (Diptera) and amphipoda, in contrast to relatively insensitive groups belonging to Diptera, Collembola, Coleoptera and Arachnida.

It is concluded that the diversity of macroinvertebrates in high altitude streams is substantial, despite the extreme conditions of this habitat. At reference sites water discharge and current velocity modulated macroinvertebrate assemblages. In natural and mine-related metal polluted streams highly correlated metal concentrations structured communities, changing their composition through replacement of sensitive taxa by more tolerant taxa.

Acknowledgements: This work was funded by the Consejo Nacional de Ciencia, Tecnología e Innovación Tecnológica del Perú (Concytec Contract 193-2006), the Fogarty International Center (NIH Research Grant # 5-D43TW005746-04) and the International Foundation for Science (Project AA19244). Raúl Loayza-Muro received a UNESCO Keizo Obuchi fellowship for writing this paper at the University of Amsterdam. We want to thank the assistance of Luis G. Chirinos, José Chang Kee, Emily Ruiz and Yajayra Vargas with insect
identifications. We are indebted to Jorge Recharte, Julio Lázaro, Teodoro Sánchez and Eusebio Salas for helping to access the sampling areas and accommodation in the field.

Supplemental data available: Table S1. This information is available free of charge via the Internet at wileyonlinelibrary.com.
Chapter 3

Metal leaching and altitude confine benthic macroinvertebrate community composition in Andean streams

Abstract

Andean streams drain metal-rich bedrock and are subjected to an extreme altitude gradient, which may create highly selective conditions for life. The aim of the present study was therefore to evaluate the simultaneous effects of metals and altitude on benthic macroinvertebrate community composition in Andean streams. Polluted sites were characterized by high metal concentrations and low pH, and high altitude sites by high ultraviolet-B radiation and low concentration of dissolved organic matter. Canonical Correspondence Analysis indicated that the patterns in faunal composition were best explained by metal pollution followed by altitude, with dipterans and collembolans occurring mostly under harsh conditions of high altitude and high metal levels. Interaction between metals and altitude was most evident at polluted sites. It is concluded that in Andean streams metal leaching from igneous rock and altitude may be important factors confining benthic macroinvertebrate communities reducing their numbers and changing their composition towards specialized taxa.
Introduction

The tropical Andes encompass vast areas with altitudes above 4000 m, creating environments that potentially challenge the survival and persistence of biota. With increasing altitude, environmental conditions, such as water temperatures, oxygen levels, nutrient concentrations and solar ultraviolet-B radiation (UV-B, 280–320 nm) become more extreme and consequently, the diversity of aquatic communities in mountain streams exhibits a decline towards the summits (Vinebrooke & Leavitt, 1999; Rostgaard & Jacobsen, 2005; Jacobsen & Marin, 2007). These correlated factors act together on alpine biota and the integrated effect may be more important than the effects of the single factors. Accordingly, vertical zonations of species distribution and variations in community structure have been better explained by altitude than by small-scale factors associated with the specific habitat (Jacobsen, 2003; Finn & Poff 2005). Yet, life at the highest altitudes (> 4000 m) under this unique suite of environmental conditions has seldomly been studied.

Environmental pollution may add further stress to life at high altitude. In the Andes metal pollution is caused by mining, but in addition, the natural weathering of metal-rich bedrock produces a continuous leaching of metals and acid drainage into streams, affecting water quality and benthic communities (Ministerio de Energía y Minas, 1998). The few studies evaluating the effects of increased acidity and metal mixtures in high altitude tropical (Loayza-Muro et al., 2010; van Damme et al., 2008) 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). Indirect effects include smothering of the streambed by metal oxyhydroxide precipitates, restricting available habitats for benthic fauna, impoverishing food quality, and modifying interactions between functional feeding groups (O’Halloran et al., 2008).

Since it remains unknown if metals and altitude shape communities in high mountains as single independent stressors or as one combined, ‘multi-stress’ selective force, the aim of this study was to examine the simultaneous effect of metals and high altitude on benthic macroinvertebrate community composition in Andean streams of the Cordillera Blanca, Peru.

Materials and methods

Study sites

In Central-Northern Peru (Ancash region), the Cordillera de los Andes comprise two parallel mountain ranges, the eastern Cordillera Blanca and the western Cordillera
Negra, which run along the Santa River. Below the permanent snow-line in the Cordillera Blanca, between 3700–4400 m a.s.l., slopes have been modified for small agriculture and cattle rearing. Streams in this area are fast flowing, with substrate consisting of gravel, pebble and cobbles in runs and riffles. They show transparent waters, a very sparse macrophyte growth and are almost completely unshaded due to the natural absence of trees or bushes growing on the banks, particularly above 4000 m a.s.l.

Eight sampling sites were selected between 3,087–4,079 m a.s.l. in the Cordillera Blanca, four being located in the Quilcayhuanca catchment and four in the Rúrec catchment (Figure 1). In both catchments a reference and a naturally polluted stream were selected at low (3,040 and 3,087 m a.s.l.) and high altitude (3,998 and 4,079 m a.s.l.). The reference sites had their source in clean lagoons or springs with a very low metal background, while the naturally polluted sites had their origin in separate gorges characterized by metal rich bedrock. All sites were sampled on the 10 and 11 of July (dry season) and on the 27 and 28 of November 2010 (rainy season), and on the 6 and 7 of March (rainy season) and on the 13 and 14 of July 2011 (dry season).
Physical chemical characteristics

Measurements of 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 were averaged. Water samples for analysis of dissolved organic carbon (DOC) were filtered through 0.45 µm membranes in the field, acidified with HCl, and stored in amber glass bottles at 4°C. The concentration of DOC was determined within 24 h using a Shimadzu TOC-5000 analyzer (Columbia, ML, U.S.A.). Stream depth was calculated from four measurements at each of three parallel cross-sections with a calibrated stick. Mean current velocity was obtained with a mechanical flow-meter (Wildlife Supply Company, Buffalo, NY, USA). Discharge was calculated as the average of the three products of mean current velocity, mean depth and stream width at three cross-sections. For determining hardness, water samples were taken with 500-mL glass bottles below the water surface, kept at 4°C in a Styrofoam box, and analyzed using standard methods (Clesceri et al., 1998). Water samples for total metals analysis were taken with 1-L polypropylene bottles, preserved with 10 N HNO₃ and analyzed by ICP-ES (induced-coupled plasma emission spectroscopy) (U.S. EPA, 1994). Samples for determining hardness and metals were taken in triplicate at each sampling site.

Since we expected the naturally polluted sites to contain a mixture of metals with potential additive effects, we calculated the cumulative criterion unit (CCU), which is a cumulative measure for all metals at a specific site, allowing examining the relationships between benthic community structure and metal concentrations (Clements et al., 2000). The CCU is defined as the ratio between the stream metal concentration and the U.S. Environmental Protection Agency (EPA) criterion value for toxicity, summing the ratios for all metals measured at a specific site (U.S. EPA, 1986): $CCU = \sum \frac{m_i}{c_i}$, where $m_i$ is the total recoverable metal concentration (dissolved and suspended fractions) and $c_i$ is the criterion value for the $i$th metal. The criterion value is based on U.S. EPA guidelines on critical concentrations, which may harm aquatic organisms when exceeded. CCU values are scaled as follows: < 1.0, no adverse effects; 1.0–2.0, adverse effects; 2.0–10.0, significant mortality to sensitive species and altered benthic community composition expected; > 10.0, extremely toxic (Clements et al., 2000). Because water hardness affects the toxicity and bioavailability of some metals, criterion values for Cd, Cu, Pb and Zn were modified according to (U.S. EPA, 1986) to account for variation in water hardness between streams.
For Al, Fe and Mn no adjustment was needed and we followed the EPA criterion values (U.S. EPA, 1986).

**Invertebrate sampling**

At each sampling site, six Surber samples (each 20 cm², mesh size 250 μm) were collected randomly, three from gravel-pebble substratum and three from stones in different microhabitats over representative sections, including stagnant water along the banks. All samples were preserved in 70% (v/v) ethanol, and sorted in the laboratory with the use of a Zeiss Stemi DV4 stereomicroscope (Göttingen, Germany). Since knowledge on Peruvian stream fauna, and South American streams in general is scarce, insects could only be identified with certainty to the family level and most non-insects to order or class, using taxonomical keys (Roldán, 1996; Domínguez and Fernández, 2009). This relatively coarse level of taxonomic resolution considers the high correlation between family richness of insects at individual stream sites and species richness, and may allow comparative analyses of community structure and detecting effects of pollution on benthic communities (Loayza-Muro et al., 2010; Vanderklift, 1996). In addition, the relative abundance of Ephemeroptera, Trichoptera and Plecoptera (%EPT) was calculated because these groups are generally considered to be sensitive to environmental pollution.

**Canonical correspondence analysis**

We selected Canonical Correspondence Analysis (CCA) based on the idea that benthic faunal assemblages most likely show non-linear relationships to the environment (ter Braak and Šmilauer, 2002). CCA was performed to examine the effects of altitude, metal pollution, and the interaction of these two factors on community composition. Prior to the CCA, the family counts were log-transformed to reduce the effects of highly abundant taxa (ter Braak and Šmilauer, 2002). The CCA analysis was performed applying default options of the CCA function in the vegan package (Oksanen et al., 2011) in R (R Development Core Team, 2011). The significance of the three canonical axes was assessed through permutation tests as implemented in the anoca.cca function of this same package, applying a maximum of 9999 permutations.

**Results**

**Characterization of sampling sites**

The UV-B radiation level at 4000 m was two-fold that at 3000 m in the dry and rainy seasons (Figure 2, Supplementary data Table 1), while water temperature, dissolved oxygen, stream discharge and water flow were similar. DOC concentration at 4000 m a.s.l.
was half that at 3000 m a.s.l. and decreased in the dry season. Conductivity and hardness were higher and pH was lower in the polluted streams compared to the reference streams. Likewise, the concentrations of all metals and CCU values were higher in the polluted streams than in the reference streams (Supplementary data Table 2), and in most of the cases they increased with altitude and from the rainy to the dry season (Figure 3). The metal concentrations at the polluted sites (e.g. Al, 4.83 mg/L; As, 0.028 mg/L; Fe, 58.8 mg/L; Mn, 1.17 mg/L; Ni, 0.11 mg/L; Zn, 0.278 mg/L) ranged from 2 (Cu) to 588 (Fe) times those at the reference sites, indicating a high degree of natural leaching. At these sites, the streambed was smothered by orange precipitates and encrusted layers, most likely comprising iron oxyhydroxides. The mean CCU ranged from 1.04 to 94.81, with all polluted sites showing values higher than 10.0, meaning potentially significant mortality to sensitive species and altered benthic community composition. Although the CCU at reference sites exceeded 1.0, indicating metal pollution, the large differences with CCU values at polluted sites allowed separating the two site categories.

Relationship between benthic macroinvertebrate community composition and physical-chemical characteristics

A total of 28 families of aquatic insects and 10 other invertebrate taxa were identified (Supplementary data Table 3). Among the insects, Diptera (11), Coleoptera (7) and Trichoptera (4) were represented by the largest number of families. The number of individuals and the number of taxa did not differ between reference sites at 3000 and 4000 m (Table 1). In contrast, the number of individuals, number of taxa, and %EPT were much lower at the polluted sites than at the reference sites. Fewest taxa survived under the harshest conditions, the polluted sites during the dry season.

The CCA ordination revealed that the patterns (Figure 4A CCA axis 1) in macroinvertebrate family composition were best related to pollution. The families Hirudidae, Oligochaeta (Annelida), Ephydridae, Chironomidae (Diptera), Ptilidae (Coleoptera), Isotomidae, Sminthuridae, Hypogastruridae (Collembola) and Acari persisted at high pollution levels (left side of CCA axis 1), suggesting a lower sensitivity towards metal contamination, and probably also to low pH. In contrast, Baetidae (Ephemeroptera), Hydrobioscidae, Hydroptilidae, Limnephilidae (Trichoptera), Dixidae, Empididae (Diptera), Scirtidae (Coleoptera), Hydracarina (Acari) and Planariidae (Turbellaria) appeared more sensitive to metal pollution, since these taxa were mostly arranged at the opposite (right) side of CCA axis 1. Altitude related mostly to the second CCA axis (Figure 4A). Heptageniidae (Ephemeroptera), Leptoceridae (Trichoptera), Blephariceridae, Simuliidae, Tipulidae (Diptera), Dytiscidae, Gyrinidae (Coleoptera) and Lymnaeidae (Gasteropoda) were more abundant better at low elevations (low side of CCA axis 2), whereas Perlidae (Plecoptera), Ceratopogonidae, Elmidae, Psychodidae, Tabanidae (Diptera), Staphylinidae (Coleoptera) and Amphipoda, Ostracoda, Copepoda (Crustacea)
Figure 2. Dot plots of the environmental properties repeatedly measured at eight sampling sites. High, 4000 m a.s.l.; Low, 3000 m a.s.l.; Pol, polluted; Ref, reference.

Figure 3. Dot plots of the metal concentrations repeatedly measured at eight sampling sites. High, 4000 m a.s.l.; Low, 3000 m a.s.l.; Pol, polluted; Ref, reference.
Table 1. Number of individuals, number of taxa, and relative abundance of individuals belonging to Ephemeroptera, Plecoptera and Trichoptera (%EPT) at the eight sampling sites in the Cordillera Blanca area, Peru. Status: Pol, polluted; Ref, reference. Months: J, July; D, December; M, March. Year: 1, 2010; 2, 2011.

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Figure 4. Canonical correspondence analysis (CCA) ordination diagrams of faunal assemblages (A: axes 1 and 2; B: axes 1 and 3) showing families (small circles) and the centroid scores of the altitude and pollution factors and their interaction (large circles), on the basis of 32 samples from eight sites (Figure 1). The eigenvalues were 0.45, 0.29, and 0.07 for axis 1, axis 2 and axis 3, respectively. All axes were significant ($p < 0.005$ for axes 1 and 2, and $p = 0.025$ for axis 3). Family codes appear in Supplementary data Table 3.
Metals and altitude confine macrofauna

persisted at high altitudes.

The third axis of the canonical ordination (Figure 4B) allowed separating the interactive effect of altitude and metals from the single effect of metals on bentic community composition. Ephydridae and Isotomidae occurred mostly at polluted sites at high altitude, whereas Sminthuridae and Ptilidae were mostly found in polluted streams at low altitudes.

Discussion

High altitude Andean streams encompass a suite of unique environmental conditions that potentially challenge the survival of aquatic biota. Indeed, the canonical ordination suggested that metals and altitude had a strong influence on bentic macroinvertebrate community composition in Andean streams.

The results of the physical chemical analyses revealed naturally occurring low pH levels and high metal concentrations at the polluted sites, which may be explained by the dominance of the Chicama formation at the upper sections containing metamorphic sedimentary rocks characterized by pyrite. Pyrite (Fe$_2$S) oxidation is the dominant reaction in the proglacial zone, generating protons and lowering the pH below 4. As a result, igneous rocks are readily weathered, resulting in high metal solute levels in the water (Burns, 2010). This may explain the high aluminum and iron concentrations in the metal-rich streams, which are present as plagioclase and biotite minerals forming the granodiorite batholith in this area. Also the highly correlated nickel, cobalt, strontium and zinc contents from the Cordillera Blanca batholiths (Rivera et al., 2008) are likely mobilized by increased acidity.

In the streams, the low pH of the leachates increased the bioavailability of the metal ions, enhancing the detrimental effects on aquatic organisms (Courtney and Clements, 2000). Also, the presence of stable orange precipitates and encrusted layers comprising iron oxyhydroxides smothered the streambed, impoverishing food and substrate quality, restricting available habitats for benthic fauna and modifying interactions between functional feeding groups (Courtney and Clements, 2000; O’Halloran et al., 2008). Metal-contaminated substrates may have negatively influenced the composition of benthic communities, producing chronic toxicity and inhibiting colonization by sensitive taxa. A similar effect has been described for several mayfly taxa preferring clean, highgradient streams with coarse substrata and a low degree of sedimentation (Courtney and Clements, 2002). The structuring role of metals was similar to that seen in other scenarios where increased metal levels and decreasing pH coincided with decreased numbers of individuals and families (Gerhardt et al., 1993; Löhr et al., 2006). Since metal concentrations are usually highly correlated in polluted environments, effects on community composition were
likely caused by all metals jointly. This was indicated by the high CCU values at the polluted sites (ranging from 11.32 to 94.81), largely exceeding the cutoff of 10.0, which represents metal mixtures causing mortality and altering community structure (Clements et al., 2000). The high CCU values were reflected by much lower numbers of individuals and taxa, and by a strong shift in family composition between reference and polluted sites. The canonical ordination identified crustaceans, ephemeropterans, trichopterans, dipterans and coleopterans as metal- and acid-sensitive groups mainly present at reference sites, and more tolerant dipterans and collembolans associated with polluted sites. This was conform our expectation, since the sensitivity of EPT taxa towards acid pH and metals has been well documented in field surveys in acid mine areas (Gerhardt et al., 2004; van Damme et al., 2008; Loayza-Muro et al., 2010) and experimental microcosms (O’Halloran et al., 2008). Although it is difficult to sort out the effect of individual metals or acid pH on the observed assemblage responses due to their strong correlation, our results suggested that the leachate of igneous rocks produced substantial shifts in community composition towards more tolerant taxa, particularly during the dry season. The differences in benthic community composition observed particularly between reference and metal-impacted streams might further be attributed to the interaction between DOM, UV-B and heavy metals, since organic matter simultaneously reduces metal bioavailability and penetration of UV-B in the water column (Kelly et al., 2001; Clements et al., 2008; Kashian et al., 2004). However, under the elevated sunlight conditions in the high Andes, it is likely that the DOM became photochemically unstable, significantly lowering metal complexation and the mitigation of metal toxicity (Brooks et al., 2007). Solar radiation also causes photobleaching of DOM degrading the chromophores that absorb light, and losing as much as half of its UVR absorption capacity (Zepp et al., 2007). Hence, the low levels of DOC in high altitude metal-impacted streams and its eventual photodegradation may have well left benthic communities more exposed to both metals and UV-B radiation.

The maximum UV-B levels registered in this study at the highest altitude sites (4.89 W/m) were most likely due to a naturally thinner ozone layer over low latitudes and the more direct path of solar radiation through the atmosphere near the equator (Kinzie et al., 1998). These values stand out exceeding those registered in temperate and high latitude alpine areas causing significant impairment of aquatic invertebrates in artificial streams [1.7 W/m² (Kiffney et al., 1997a); 2.7 W/m² (McNamara and Hill, 1999)], and those observed to structure natural invertebrate communities in experimental field studies [0.1 W/m² (Cabrera et al., 1997); 0.17 W/m² (Vinebrooke and Leavitt, 1999); 0.5 W/m² (Kiffney et al., 1997b); 1.6 W/m² (Kelly et al., 2003)]. Since these values are among the highest irradiances reaching the Earth’s crust, they may be well considered an important driver of the altitudinal responses of benthic assemblages observed in the present study. Intense radiation may inhibit sensitive taxa either directly or indirectly by altering food resources (Kiffney et al., 1997b; Kelly et al., 2001). Simuliidae, for example, were abundant during the dry
season only at low altitude sites, which agrees with previous studies describing a strong drift response and emigration of blackflies due to high UV exposure (Donahue and Schindler, 1998; Kelly et al., 2001). On the other hand, the presence of crustaceans in the reference high altitude streams may be related to the accumulation of photoprotective pigments, such as carotenoids and mycosporine-like amino acids (Rautio et al., 2009). These substances are effective solar radiation screeners providing protection against the harmful effects of UV-B radiation, especially in shallow UV-transparent water bodies as those found in the Andes at 4000 m a.s.l.

Persistence of species under high solar radiation may also be based on avoiding the high UV-B levels, related to preferences for habitats providing considerable shading, such as sediment dwelling in the case of midges or opaque case-protection for caddisflies. Similarly, aquatic vegetation may provide physical refuge from elevated UV-B radiation (Vinebrooke and Leavitt, 1999). Indeed, ephemeropterans, coleopterans and amphipods were abundant at the high altitude reference site in Quilcayhuanca, where macrophyte cover provided food and shading, and low stream discharge and flow provided also habitat stability allowing an important number of individuals and taxa. Although macrophytes may have obscured part of the blazing UV at this high site, the response of specific taxa suggests that high UV-B conditions may be considered a relevant factor producing changes in assemblage composition, particularly during the dry season coinciding with low water flow, shallow-depths and low DOC content in the water.

The altitudinal response of the benthic communities might also be partially due to stream velocities. The high altitude streams, where water flow and discharge levels were relatively low, were dominated by Amphipoda, Ostracoda, Copepoda (Crustacea), Perlidae (Plecoptera) and Elmidae (Coleoptera). Similar responses of these taxa have been recorded in other invertebrate communities (Miserendino and Pizzolón, 2003; Scheibler and Debandi, 2008).

The CCA analysis suggested that the altitudinal effect on family composition at polluted sites differed from that at reference sites. Ephydridae and Isotomidae, the taxa surviving only under harsh conditions of high altitude and high pollution levels, have been described to occur in inhospitable environments, such as alkaline or saline lakes, hot springs, crude oil pools and mud flats exposed to ultraviolet radiation (Foote, 1995; Wagner et al., 2008), and metal polluted soils (Crouau and Pinelli, 2008), respectively. They were also present at high-altitude and metal-polluted streams elsewhere in the Peruvian Andes (Loayza-Muro et al, 2010). The altitudinal effect occurring at polluted sites is probably explained by the differences in streamed composition between altitudes. At high altitudes, mud-shore habitats are typical along the margins of polluted streams and may well represent a rich food supply supporting a large array of Ephydridae (Foote, 1995), while at 3000 m polluted streams show substrates consisting of stones, pebbles and cobbles. In
contrast, reference streams showed a more homogenous stony substrate composition at both altitudes.

The aim of the present study was to evaluate the simultaneous effect of metal pollution and altitude on the composition of benthic macroinvertebrate communities in Andean streams. Our results showed that the faunal composition was mostly related to pollution followed by altitude. An interaction of these factors was most evident at polluted sites, with dipterans and collembolans occurring mostly under harsh conditions of high altitude and high pollution levels. Hence it is suggested that in highland Andean streams metal pollution and altitude modulate benthic macroinvertebrate assemblages, reducing their numbers and changing their composition towards specialized taxa.

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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.](image-url)
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 HNO₃ 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 H₂O₂ (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 H₂O₂ (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 H₂O₂ (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$_2$·2H$_2$O, 180 mg/L MgSO$_4$·H$_2$O, 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% HNO₃. 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% HNO₃ was added and the samples were placed in a 100 °C heat block for 2.5 h. Next, 100 µL 65% HNO₃ 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 reference 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>
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<th>Status</th>
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<th>Conductivity (μS/cm)</th>
<th>pH</th>
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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.](image)

**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 \((n = 3, r = 0.98, p = 0.001; \text{Figure } 3B)\) and melanin \((n = 3, r = 0.95, p = 0.009; \text{Figure } 3C)\) 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 occurred (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/m²) compared to the highest sunlight regimes at 4000 m in the Andes (5.23 W/m²).

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).

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Science and Technology of Peru (FINCyT, Contract No. 113-2009-FINCyT-BDE) for writing this paper at the University of Amsterdam.

Supporting information: Table S1. This information is available free of charge via the Internet at http://pubs.acs.org.
Chapter 4
Chapter 5

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

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/m², 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.

**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 triplicate. 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’-GGTCAACAAATCATAAAGATATTTGG-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 H2O, 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 H2O2 (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 H2O2 (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 H2O2 (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 Quilcayhuanc 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/m²) at the highest sites at 4000 m. Water pH ranged from 6.4 to 8.5, temperature from 9.7 to 10.3°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.

<table>
<thead>
<tr>
<th>Catchment</th>
<th>Season</th>
<th>Altitude (m)</th>
<th>UV-B (W/m²)</th>
<th>Conductivity (µS/cm)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Oxygen (mg/L)</th>
<th>DOC (mg/L)</th>
<th>Depth (cm)</th>
<th>Discharge (L/s)</th>
<th>Velocity (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quilcayhuanca</td>
<td>Dry</td>
<td>3200</td>
<td>2.23</td>
<td>73</td>
<td>6.5</td>
<td>13.7</td>
<td>5.2</td>
<td>0.99</td>
<td>89</td>
<td>410</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4000</td>
<td>4.89</td>
<td>70</td>
<td>7.4</td>
<td>12.4</td>
<td>5.6</td>
<td>0.52</td>
<td>56</td>
<td>350</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Rainy</td>
<td>3200</td>
<td>1.08</td>
<td>29</td>
<td>7.3</td>
<td>10.0</td>
<td>5.9</td>
<td>1.26</td>
<td>101</td>
<td>640</td>
<td>62</td>
</tr>
<tr>
<td>Rímac</td>
<td>Dry</td>
<td>650</td>
<td>0.84</td>
<td>479</td>
<td>8.4</td>
<td>16.3</td>
<td>5.2</td>
<td>3.35</td>
<td>99</td>
<td>600</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1900</td>
<td>1.21</td>
<td>349</td>
<td>8.3</td>
<td>14.5</td>
<td>5.6</td>
<td>2.11</td>
<td>82</td>
<td>520</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3200</td>
<td>2.35</td>
<td>254</td>
<td>8.5</td>
<td>12.2</td>
<td>5.9</td>
<td>1.21</td>
<td>73</td>
<td>460</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Rainy</td>
<td>650</td>
<td>0.49</td>
<td>313</td>
<td>8.0</td>
<td>15.1</td>
<td>5.5</td>
<td>4.05</td>
<td>117</td>
<td>810</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1900</td>
<td>0.77</td>
<td>277</td>
<td>7.8</td>
<td>12.2</td>
<td>5.8</td>
<td>2.97</td>
<td>106</td>
<td>730</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3200</td>
<td>1.33</td>
<td>168</td>
<td>7.9</td>
<td>11.0</td>
<td>6.1</td>
<td>1.82</td>
<td>98</td>
<td>650</td>
<td>68</td>
</tr>
</tbody>
</table>
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 ($P < 0.05$).

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Figure 3. Phylogenetic relationships of Baetidae taxa from the Quilcayhuanca River and the Rimac 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/m² 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-overlapping 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 cannot 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 defenses in aquatic invertebrates including behavioral 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 behavior 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 demonstrate 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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Chapter 6

Metals and altitude drive genetic diversity of chironomids in Andean streams

Abstract

Andean streams cover steep altitude gradients and locally leach metal-rich bedrock, creating highly selective conditions for life. Chironomids are among the few dominant insect taxa present under the harshest conditions in Andean high altitude streams. Yet, the question remains if their dominance is due to either an adaptive capacity of few species (population differentiation) or to a diversity of species with different capacities to cope with environmental extremes (species composition). Therefore, the aim of the present study was to assess if metals and altitude drive the genetic diversity of chironomids in Andean streams.

We measured metal concentrations and UV-B radiation in reference and polluted streams located at both 3000 and 4000 m above sea level (a.s.l.). The genetic composition of the chironomid communities from these streams was determined by mitochondrial cytochrome oxidase I (COI) gene sequencing and a phylogenetic tree was constructed.

The concentrations of all metals were higher in the polluted streams than in the references streams, while the UV-B radiation level at 4000 m was circa 50% higher than at 3000 m. At 3000 m the reference site was inhabited by 6 phylogenetic species, completely different from the 3 present at 4000 m. Only one common phylogenetic species was present at the metal-rich sites at 3000 and 4000 m, which did not occur at the reference sites.

The differences in phylogenetic species between 3000 and 4000 m indicated a strong sorting of species according to altitude. However, the unique phylogenetic species present at the metal-rich sites both at 3000 and 4000 m indicated that the extreme selection pressure by metal exposition overruled altitude driven selection. It is concluded that altitude limits the distribution of chironomid taxa, yet, metal selection leads to predominance of a unique metal tolerant taxon.
Introduction

Species inhabiting high altitude streams are exposed to a suite of harsh environmental conditions, such as low water temperatures, low oxygen levels and a blistering solar radiation, which challenge the survival and persistence of aquatic biota (Cabrera et al., 1997; Jacobsen and Marin, 2007; Jacobsen, 2008). Especially Andean high altitude streams above 3500 m are exposed to intensive ultraviolet-B radiation (UV-B, 280–320 nm) due to the thinner ozone layer over low latitudes and the more direct path of solar radiation through the atmosphere near the equator (Villafañe et al., 1999). This is accentuated by little riparian canopy providing hardly any shade and by high water transparency due to low levels of dissolved organic matter, allowing the penetration of biologically active UV radiation (Laurion et al., 2000; Kelly et al., 2001; Clements et al., 2008). Indeed, the maximum UV-B levels in this region, particularly in the dry season (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 mountain areas (Kiffney et al., 1997a; Kiffney et al., 1997b).

Metal pollution may add further stress to life at high altitude, since in the Andes, mining as well as natural weathering of metal-rich bedrock produce a continuous leaching of metals and acid drainage into streams, affecting water quality and benthic communities (Loayza-Muro et al., 2010). The few studies evaluating the effects of increased acidity and metal mixtures 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).

Under the harshest environmental conditions, polluted sites at the highest altitude in the Andes, chironomids are among the few persisting species (Loayza-Muro et al., unpublished; Loayza-Muro et al., 2010). Yet, the question remains if the persistence of chironomids in metal polluted Andean high altitude streams is attributable to population differentiation (i.e. species turnover) or to changed species composition (i.e. interspecific differences). Answering this question is, however, hampered by the unreliable morphological identification of chironomid species, partly due to the low resolution of current taxonomical keys for the Andes. Therefore, the aim of the present study was to assess if metals and altitude drive the genetic diversity of chironomids in Andean streams. To attain this goal, chironomids were sampled from reference and metal polluted streams at 3000 and 4000 m in the Peruvian Andes. The genetic composition of the chironomid communities from the different sampling sites was determined by mitochondrial cytochrome oxidase I (COI) gene sequencing and the construction of a phylogenetic tree.
Chapter 6

Materials and methods

Study sites

Four sites in the Quillcayhuancan catchment in the Cordillera Blanca mountain range in the Peruvian Andes were sampled (Figure 1). A clean site and a polluted site were located at both 3000 and 4000 m a.s.l, respectively. Samples were collected in January and February 2012.

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 HD 2302.0 photo-radiometer and a LP 471 cosine-corrected broad-band UVB sensor, with a maximum detection at 305 nm (Delta Ohm, Padua, Italy). UV-B irradiance was measured every second day during January and February, from 10:00 h to 14:00 h under full sun, and the maximum values obtained during each month were averaged. Water samples for total metal analysis (Al, As, Ca, Co, Cu, Fe, Mn, Ni, Sr, Zn) were taken in triplicate at each sampling.
Metals and altitude drive genetic diversity

Site with 1-L polypropylene bottles, preserved with 10 N HNO₃ and analyzed by ICP-ES (induced-coupled plasma emission spectroscopy; Varian Liberty 100, USA). Quality control was carried out by analyzing USEPA blanks and reference material for water samples.

Invertebrate sampling

Chironomid larvae were collected from gravel-pebble sediments and stones along the banks, using a set of sieves and plastic trays. Animals were transported in plastic jars to the laboratory, sorted individually in 1.5 mL screw cap tubes containing 100 µL of TRIzol reagent (Invitrogen, USA), homogenized with a plastic pestle and stored at -80°C until shipping to the University of Amsterdam (Netherlands).

DNA extraction, PCR and sequencing

For DNA extractions, 20 µL of chloroform was added to the homogenate. Samples were then incubated at room temperature (RT) for 5 min and centrifuged at 12000 g for 15 min at 4°C. The overlying aqueous phase containing RNA was removed and saved at -80°C for further analysis. 30 µL of TNES-6U buffer (10 mM Tris–HCl, pH 8.0; 125 mM NaCl; 10 mM EDTA; 1% sodium dodecyl sulfate [SDS]; 6 M urea) was then added to the phenol-chloroform phase and incubated at RT for 10 min. Subsequently, samples were centrifuged at 18000 g for 15 min at 4°C and the upper aqueous phase was transferred to a clean tube. An equal volume of 100% isopropanol was added and samples were incubated at -80°C overnight, after which they were centrifuged at 18000 g for 30 min at 4°C. The supernatant was removed and pellet was washed 1 or 2 times with 80% ethanol. DNA was then eluted in 20 µL of TE buffer and stored at -20°C.

For amplification and sequencing of COI, DNA was taken from -20°C, thawed and, if necessary, diluted to a final concentration of 5-10 ng DNA/µL. A ± 709 bp fragment of the COI gene was amplified using primer 911 (forward, 5’-TTT CTA CAA ATC ATA AAG ATA TTG G-3’) and 912 (reverse, 5’-TAA ACT TCA GGG TGA CCA AAA AAT CA-3’) (Folmer et al. 1994). Amplifications were performed in a 20µL reaction volume consisting of 8.2 µL H₂O, 4.0 µL 5X PCR buffer, 4.0 µL 1mM dNTP’s, 0.6 µL 10 mg/mL bovine serum albumin, 0.4 µL 10µM of each primer, 0.4 µL 5U/µL “Phire Hot Start II” polymerase (Finnzymes, Finland) and 2.0 µL of template DNA. PCR cycling conditions were: initial denaturation at 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 55 °C for 10 sec and elongation at 72°C for 20 sec, and a final elongation step at 72°C for 5 min followed by 5 min cooling down at 4°C. PCR products were put on a 1% agarose gel to determine their quality and size. A mixture of 1µL PCR product and 1µL of either forward or reverse primer with 7µL H₂O was then sent to MacroGen Europe (Amsterdam, Netherlands) for sequencing. The returned sequences were trimmed to the same length based on quality scores. Sequences were then translated using
the invertebrate mtDNA codon table to determine that they contained no stop codons, which is evidence that they are genuine mtDNA sequences and not NUMTS (Buhay, 2009; Moulton et al., 2010). A BLASTn search in GenBankTM was then conducted to confirm that the sequences were from chironomids. Next, our COI sequences were aligned by clustal W embedded in MEGA 5.0 (Tamura et al., 2011). A model test embedded in MEGA 5.0 was run to determine the best fitting nucleotide substitution model.

Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 531 positions in the final dataset.

Results

Physical chemical characteristics

The UV-B radiation level at 4000 m was circa 50% higher than at 3000 m. Water temperature was lower at 4000 m than at 3000 m, while dissolved oxygen showed no evident differences between altitudes (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 (Table 2). The metal concentrations at the polluted sites (e.g. Al, 1.69 mg/L; As, 0.011 mg/L; Cu, 0.087 mg/L; Fe, 3.2 mg/L; Mn, 0.644 mg/L; Ni, 0.026 mg/L; Zn, 0.243 mg/L) ranged from 2 (As) to 322 (Mn) 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 chemical variables at the four sampling sites in the Cordillera Blanca area, Peru. Status: Ref, reference; Pol, polluted.

<table>
<thead>
<tr>
<th>Altitude</th>
<th>Status</th>
<th>UV-B (W/m²)</th>
<th>Conductivity (µS/cm)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Oxygen (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000 m</td>
<td>Ref</td>
<td>2.31</td>
<td>73</td>
<td>8.0</td>
<td>15.0</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>Pol</td>
<td>1.92</td>
<td>155</td>
<td>4.5</td>
<td>14.3</td>
<td>6.8</td>
</tr>
<tr>
<td>4000 m</td>
<td>Ref</td>
<td>3.06</td>
<td>50</td>
<td>7.0</td>
<td>8.2</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Pol</td>
<td>2.95</td>
<td>132</td>
<td>4.3</td>
<td>9.3</td>
<td>5.1</td>
</tr>
</tbody>
</table>
Table 2. Mean metal concentrations (mg/L) at the four sampling sites in the Cordillera Blanca area, Peru. Highest/lowest metal concentration indicates the ratio between the highest and the lowest mean metal concentration. Status: Ref, reference; Pol, polluted.

<table>
<thead>
<tr>
<th>Altitude</th>
<th>Status</th>
<th>Al</th>
<th>As</th>
<th>Ca</th>
<th>Co</th>
<th>Cu</th>
<th>Fe</th>
<th>Mn</th>
<th>Ni</th>
<th>Sr</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000 m</td>
<td>Ref</td>
<td>0.43</td>
<td>0.06</td>
<td>6.8</td>
<td>0.001</td>
<td>0.003</td>
<td>0.7</td>
<td>0.011</td>
<td>0.001</td>
<td>0.050</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Pol</td>
<td>1.66</td>
<td>0.009</td>
<td>12.1</td>
<td>0.014</td>
<td>0.082</td>
<td>2.3</td>
<td>0.561</td>
<td>0.026</td>
<td>0.088</td>
<td>0.243</td>
</tr>
<tr>
<td>4000 m</td>
<td>Ref</td>
<td>0.07</td>
<td>0.007</td>
<td>8.8</td>
<td>0.001</td>
<td>0.003</td>
<td>0.4</td>
<td>0.002</td>
<td>0.001</td>
<td>0.032</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Pol</td>
<td>1.69</td>
<td>0.011</td>
<td>15.7</td>
<td>0.020</td>
<td>0.087</td>
<td>3.2</td>
<td>0.644</td>
<td>0.022</td>
<td>0.077</td>
<td>0.211</td>
</tr>
<tr>
<td>Highest/lowest Metal conc.</td>
<td>24</td>
<td>2</td>
<td>2</td>
<td>20</td>
<td>29</td>
<td>16</td>
<td>322</td>
<td>26</td>
<td>4</td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

**Phylogenetic tree and species distribution**

The following number of individuals was collected at the sampling sites: reference, 3000 m = 20; polluted, 3000 m = 50; reference, 4000 m = 208; polluted, 4000 m = 50. Sequencing and analysis of a portion of the mitochondrial COI gene resulted in a final dataset of 531 bp for 81 individual chironomid larvae collected at the four sampling sites.

There is no taxonomic reference available for chironomids in the Andes. In addition, most specimens were collected as larvae, which are even more difficult to identify to the species level. Therefore, we used phylogenetic species as a proxy for species identity, adopting this species concept (Nixon and Wheeler, 1990) as our working hypothesis. Phylogenetic species were considered as different species when the genetic distance between them exceeded 2%. This resulted in the distinction of 10 species. The mtDNA COI sequences of chironomids that are available in Genbank and IBOL are mostly from specimens collected from North America, Europe and Australia. Indeed, BLAST searches with the Andean phylogenetic species did not return identical or near perfect matches, indicating that most of them probably represent new species not yet morphologically described.

The Maximum Likelihood phylogenetic tree was inferred using the General Time Reversible model with a Gamma distribution (5 categories (+G, parameter = 1.4681)) (Figure 2). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 63.2768% sites). The mean p-distance between samples was 0.118, while within the clades of parenthesis average p-distance was less than 0.02. The value of 0.118 is well above 0.02-0.3, which is often used as a cut-off for the distinction of phylogenetic species. Thus, all terminal branches longer than 0.02 are probably indeed different species. The phylogenetic tree shows a distinct grouping of phylogenetic species by their sampling site. The upper cluster consists of near identical phylogenetic species originating from the polluted sites at 3000 and 4000 m. Below that cluster, there is a single phylogenetic species...
Figure 2. Phylogenetic relationships of chironomid taxa collected at reference and polluted sites at 3000 and 4000 m. The best fitting nucleotide substitution model used for inferring the maximum likelihood tree based on a 709 bp COI mtDNA sequence of 81 individuals was the GTR+G. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown above the branches. Only values greater than 90% are shown. 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.
corresponding to the 4000 m reference site and a more diverse cluster consisting of phylogenetic species from the 3000 m reference site. The lower part of the tree consists of one phylogenetic species from the 4000 m reference site, a small cluster consisting of two phylogenetic species from the 3000 m reference site and a large cluster consisting of a single phylogenetic species from the 4000 m reference site.

An overview of the number of chironomid phylogenetic species per sampling site is given in Figure 3. At 3000 m the reference site was inhabited by 6 species and the polluted site only by one, both sites having no species in common. At 4000 m the reference site was inhabited by one species with a high abundance and one representative of two different species. At the polluted site at 4000 m one species was present, again both sites having no species in common. At the metal-rich sites the same species was found at both altitudes, contrasting with the completely different set of taxa found at the reference sites. Yet, the two reference sites also had no species in common, the 4000m site being less diverse than the 3000 m site.

**Discussion**

The phylogenetic species identified in the present study in the Andes probably all represent new species, not yet morphologically described. Moreover, the phylogenetic analysis enabled us to identify separate groups of chironomids distributed among reference and polluted streams at 3000 and 4000 m, revealing that chironomid species composition...
was altitude and pollution level specific. The reference sites exhibited a higher and completely different species diversity compared to the polluted sites at both altitudes, which suggests that the chironomids at the polluted sites were subjected to a selective pressure by elevated metal concentrations. Moreover, at 4000 m, the distance between the reference and the polluted site was only 200 m. Hence a high dispersal and oviposition of all species at both sites was likely to take place, but this did not result in a single common species, confirming that the metals acted as a selective force at the polluted sites, eliminating sensitive species. The presence of only one species in metal-rich streams may indicate a strong reduction of genetic variability, as previously described for populations of invertebrates inhabiting sites heavily contaminated by metals (van Straalen & Timmermans, 2002; Fratini et al., 2008; Ungherese et al., 2010).

The presence of a chironomid species under the most extreme conditions, the metal-polluted high altitude site, is in agreement with the occurrence of Chironomidae in acidified metal-polluted temperate (de Haas et al., 2005; Janssens de Bisthoven, Gerhardt & Soares, 2005) and tropical high altitude streams (Smolders et al., 2003; Lohr et al., 2006; Loayza-Muro et al., 2010), and pristine glacier fed high altitude streams (Hamerlík & Jacobsen, 2012). Physiological adjustments may be responsible for such tolerance, since Chironomus species from contaminated sites are better capable of regulating the body concentration of metals compared to other taxa (Krantzberg & Stokes, 1989). Recently, it was observed that chironomids from higher altitudes and from polluted sites contained more melanin than species from reference and from lower sites (Loayza-Muro et al., 2013). This suggests that in chironomids melanin may function both as a UV-B radiation protector and metal chelator, which could well be the case in the single species present in the metal-polluted high altitude stream. Genetic adaptation has also been considered as a mechanism of metal tolerance in Chironomus species from heavily polluted environments (Groenendijk et al., 2002; van Straalen et al., 2005; Buchwalter et al., 2008). Yet, the adaptation of this single chironomid species to the elevated metal concentrations may have come with direct costs represented by smaller individuals compared to those from species in nearby reference streams. Allocation of energy towards tolerance mechanisms, such as metal-binding metallothioneins (Gillis, Reynoldson & Dixon, 2006), melanin production or cuticle sclerotization in chironomids (Loayza-Muro et al., 2013), may convey a trade-off evidenced in a reduced growth (Sibly & Calow, 1989).

The chironomid community from the reference stream at 4000 m was less diverse than the one from 3000 m and both sites had no species in common, again despite the potential for dispersal of adults, larval drift and subsequent gene flow along the same catchment. This suggests adaptation to specific altitude related environmental conditions and that species separated by altitude may possess different traits, such as feeding habits, respiration capacity, body size, adaptation to current flow and attachment to substratum.
related to habitat attributes, as observed for benthic macroinvertebrate assemblages in Bolivian high altitude streams (Tomanova & Usseglio-Polatera, 2007).

It is concluded that altitude imposes strict limits to the distribution of chironomid taxa creating a strong vertical zonation on the slopes of the high Andes, yet, selection in acidic metal-rich waters is so strong that altitude driven selection is overruled, leading to predominance of a unique metal tolerant taxon.

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Chapter 7

Concluding remarks
In the General Introduction I depicted aquatic biota in high altitude Andean streams as “life at the edge” and here I would like to reflect on this metaphor. First, the present thesis proved the highly selective force of altitude, showing that the highest sites are inhabited by a unique fauna, as discussed below. The next question was which mechanisms allow these invertebrates to maintain viable populations under conditions that are fatal to so many others. Several adaptation strategies can be envisaged, but this thesis focussed on melanin, and showed that this pigment may protect invertebrates against the adverse effects of exposure to both UV radiation and metals. Melanin synthesis and other traits enabling fauna to cope with life at high altitude are discussed in the second paragraph. The two main factors identified in the present project to drive the composition of benthic invertebrate assemblages in Andean streams were altitude and metals. But do they shape communities as single stressors or do they join into one combined selective force, ‘multi-stress’? This will be discussed in the third paragraph. Finally, the conclusions will be drawn concerning the drivers of the unique fauna inhabiting pristine and metal-rich high altitude Andean streams.

Benthic invertebrates inhabiting high altitude Andean streams: a unique fauna

The uplift and mountain building of the Andes during the late Miocene (~12 Ma) entailed a series of climatological and geological processes, such as glaciations and the closing of the Panama Isthmus, that ultimately drove the diversification of South American fauna (Hoorn, 2010) in a notably different way from that occurring in the Pyrenees, the Alps or the Himalayas (Illies, 1969; Jacobsen et al., 1997). As a result, strong selection and different dispersion mechanisms of each faunal group, modified by regional (e.g. altitude) and local (e.g. physical and chemical) factors, have determined the present day distribution of South American aquatic fauna (Covich, 1988; Sites et al., 2003). In recent years there has been an increasing interest in exploring the Andean benthic fauna, focusing mostly on the Northern (Colombia and Ecuador; Jacobsen, 2008 and references therein) and Southern (Argentina and Chile; Rodrigues Capítulo et al., 2001; Scheibler and Debandi, 2008; Miserendino, 2001; Miserendino and Pizzolón, 2003) regions, but leaving the Central (Bolivian and Peruvian) Andes almost unstudied (Jacobsen and Marin, 2007; Tomanova and Usseglio-Polatera, 2007; Acosta, 2009). This thesis is among the first studies to provide this knowledge by describing the macroinvertebrate fauna in high altitude Andes (chapters 2 and 3) and identifying for the first time the mayfly genera *Thraulodes, Americabaetis, Dactylobaetis* and *Tupiara* as well as the genetic diversity of chironomid communities using COI sequencing (chapters 5 and 6).

It is generally accepted that invertebrate taxa richness decreases with increasing altitude, mostly because the species’ temperature and oxygen tolerance limits are approached, as seen for Plecoptera, Coleoptera, Heteroptera and Odonata (Illies, 1969;
Jacobsen et al., 1997; Jacobsen, 2004). Yet, in the present study a total number of 55 taxa was identified at 4000 m, in accordance with or even higher than data on invertebrate assemblages from other Andean streams at similar altitude ranges in Ecuador (29–60) (Jacobsen, 2008; Monaghan et al., 2000), Peru (40) (Acosta, 2009), and Bolivia (26) (Jacobsen and Marín, 2007). The major groups of benthic invertebrates, such as Ephemeroptera, Plecoptera, Trichoptera, Diptera, and Coleoptera were well represented among the reference streams, with Baetidae, Perlidae, Limnephilidae, and Chironomidae being the dominant families. The families Hirudidae, Oligochaeta (Annelida), Ephydridae, Chironomidae (Diptera), Ptilidae (Coleoptera), Isotomidae, Sminthuridae, Hypogastruridae (Collembola) and Acari persisted at high pollution levels, while Perlidae (Plecoptera), Ceratopogonidae, Elmidae, Psychodidae, Tabanidae (Diptera), Staphylinidae (Coleoptera) and Amphipoda, Ostracoda, Copepoda (Crustacea) persisted at high altitudes.

Because knowledge of Peruvian stream fauna and South American streams in general is scarce, in the chapters 2 and 3 the taxa could only be identified with certainty to the family level. This came with limitations: in chapter 5 individual mayfly larvae had to be pooled for reliable melanin analysis, but the identity of the species making up the above mentioned families remained obscure, and consequently I could not answer the question if altitude-related differences in melanin concentration were due to changes in community composition or to population differentiation in mayfly species. The latter motivation also held for the chironomid communities, which were present at all sampling sites (chapter 6). Therefore, we decided to progress from traditional morphology-based taxonomy towards genetic identification. Our results showed that the sequenced mayflies and chironomids belonged indeed to these groups, but did not match with known species in Genbank, mainly originating from studies of North American, European and Australian species, while South American species are lacking. These results indicated that most Andean haplotypes probably represent new species not yet morphologically described. Moreover, for two mayfly genera and the chironomid species it was observed that sites at different altitudes and pollution status were inhabited by entirely different species. Thus the benthic invertebrate assemblages in high altitude Andean streams are highly diverse and probably consist of several new species. This unique fauna may have developed during the uplift of the Andean mountain range, yet the present day extreme conditions on these tropical mountains urge for an analysis of factors driving the distribution of species.

**Faunal traits to cope with life at high altitude**

The development of a unique fauna in streams of the tropical high Andes implies that this fauna has developed traits that provide unique adaptations to local landscapes and local climate. However, even the fauna at the highest altitudes- above 4000 m- comprise many species belonging to diverse taxonomic groups, such as discussed above. Therefore, the faunal traits involved may be equally diverse.
Tomanova and Usseglio-Polatera (2007) suggested that in high altitude Andean streams in Bolivia larval traits, such as feeding habits, respiration activity, maximum body size, adaptation to current flow, and mobility and attachment to substratum, were significantly differing from those reported for temperate and lower areas. But also traits of the flying adults may be at play. Mayflies, caddisflies and chironomids depend on bridal flights depending on local weather and landscape characteristics, especially trees (Finn and Poff, 2008). At altitudes around 4000 m vegetation is sparse though, while at 3000 m streams may pass through mountain forests. The observed distinct differences in mayfly (chapter 5) and chironomid communities (chapter 6), representing a strong altitude zonation, may thus be caused by the prominence of species that either exploit leaf detritus produced by tree species (larval traits), or the support of trees and shrubs for their bridal flight (adult traits) or both.

Oxygen in conjunction with temperature has been considered as a master factor for the evolution of insects in streams (Resh and Rosenberg, 1984). The temperature stability of cool, groundwater fed streams and their equally stable high oxygen concentration has been brought forward as a precondition for an evolutionary diversification of riverine insects such as mayflies and stoneflies (Vannote and Sweeney, 1980). Indeed experimental proof was provided for a limited tolerance of several riverine insect larvae to oxygen concentrations below air saturation in temperate areas (van der Geest et al., 2002), and has also been verified under the extremes of the tropical Andes (Rostgaard and Jacobsen, 2005; Jacobsen and Brodersen, 2008). It is remarkable though that the species-rich mayflies, abounding in well oxygenated streams, are also well represented at altitudes over 4000 m in the Andes where the oxygen level is only half of that at sea level. Moreover, the little oxygen that is present may be activated by light (Souza et al., 2007), even more so by the abundantly present UV radiation, resulting in reactive oxygen species (ROS) that damage cellular processes (Meng et al., 2009). Thus traits of the high altitude fauna in response to oxygen are likely to involve their capacities to cope with low levels high up in the mountains, but meanwhile also their capacity to deal with oxidative stress.

All the mechanisms described above, likely to operate in the high Andes, have not been substantiated yet. A notable trait described in this thesis is melanization of benthic invertebrate taxa producing a vertical zonation of pigmentation. Melanization is a first line defense mechanism described mostly in planktonic crustaceans and serving against the harmful effects of intense sunlight (Sommaruga, 2010). However, the present study is the first to describe this pigmentation in benthic macroinvertebrates in high altitude streams, and it was also commonly found in mayflies and chironomids (chapters 4 and 5). It is tempting to compare pigmentation of the high altitude fauna solely to that of sun-exposed crustaceans at sea level and conclude on the common traits of the invertebrate fauna. The discovery of an even more strong pigmentation in chironomids in metal polluted sites at
3000 and 4000 m altitude revealed an alternative role of the ‘sun screen’ melanin i.e. sequestering of metals and protection against metal toxicity (chapter 4). Now I propose the capacity to synthesize melanin as a trait that is highly effective for insect species in the environment of the high Andes, where quite commonly pockets of metal-rich rock are eroded by water, and a scorching UV radiation dominates upon all the tropical Andes. The example of the trait ‘melanin synthesis’, conveying a two-fold tolerance, may indicate that also for the other challenges for fauna described above (response to oxygen/ROS and exploiting leaf litter or flight zones provided by trees) multiple roles may be associated to seemingly pinpointed traits.

Is life at high altitude determined by single or multiple stressors?

A stressor is defined as a factor, either natural or human-caused, that drives species and populations beyond conditions normally experienced throughout their life history, potentially altering community structure and function, and thus provoking a measurable biological or ecological response (Calow, 1989, Statzner, 2010). In nature, the fitness of species may be affected by a single stressor, e.g. a heat wave, or by multiple stressors, e.g. heat, toxicants and food shortage acting together (Folt et al., 1999; Heugens, 2001). Joint effects of stressors may potentially be weaker than predicted from single action, they may be antagonistic, or may be stronger than expected or synergistic. In the present study I have observed primarily community composition, a feature that emerges from the selection of individual species under local conditions. Multiple stressors are likely to be altitude bound UV radiation and oxygen regime, while at some places increased metal exposure was evident. In the most extreme cases of metal polluted streams at 3000 and 4000 m high the community of invertebrates was reduced to 4-8 species (chapter 3) and to a single abundant chironomid taxon (chapter 6). These reduced species numbers are probably the result of few available species capable of persisting under the local multi-stress. It was argued in chapter 6 that metal contamination was so strong to overrule any effect of altitude on chironomid taxa, and in chapter 3 I brought forward that altitude and metal contamination did not interact with the distribution of all invertebrates identified at the genus level. Yet it can also be argued that the altitudes sampled (3000 and 4000 m) do not encompass the reference of lowland UV and oxygen regimes. Thus the stress factor ‘metals’ cannot be regarded as a single condition selecting the few surviving invertebrate taxa. Metals leaching from rock provide also intrinsically ‘multi-stress’: seven metals (with well-known differences in toxicity and modes of action) were elevated >16x at polluted sites (chapter 6), while pH values < 4.5 and a greatly modified water hardness are known to modify metal availability and toxicity (Gerhardt, 1993; Clements et al., 2000).

At reference sites on the Andean slopes between 3000 and 4000 m I observed a very strict zonation of putative species of chironomids (chapter 6) and also genetic characteristics of five genera of the mayfly family Baetidae were distributed over specific
altitude ranges between 650 and 4000 m (Chapter 3). These distribution patterns were linked to the single factor ‘altitude’, but certainly this factor is composed also of a multitude of individual parameters, including UV radiation, oxygen regime, scouring currents and food availability. The last factor (food) is not commonly regarded as a stress factor, but food shortage is so and food supply in mountain streams is subject to (episodic) disturbance (Tomanova et al, 2006). Indeed, the common ability of generalist macroinvertebrates to exploit changing resources in tropical streams may potentially contribute to the maintenance of population stability against natural fluctuations (Hart and Robinson, 1990).

In conclusion it is argued that ‘multi-stress’ rules the selection and distribution of invertebrate species at all altitudes in the Andes, but nevertheless is most evident at high altitude and high metal exposure: at ‘the edges of life’.

**General conclusions**

High altitude Andean streams harbor a quite diverse benthic community, represented by the major groups of invertebrates. Evidence is provided that abundant insect species have not been described taxonomically and that unique genotypes occur, probably as a result of the geological history of the Andes and the strong selection for high altitude tolerance. Cuticular pigmentation of larvae was demonstrated to form an inducible sunscreen against scorching UV radiation, but the pigment melanin was also effective in mitigating metal stress. Thus a single trait of the fauna enabled species to cope with the combined stressor UV radiation and metal exposure from leaching rock. The only species from the stress tolerant chironomid that survived the most harsh condition of a metal-rich, UV blasted site was a non-identifiable chironomid, characterized as a new haplotype. Most likely the vertical zonation of insect fauna on the slopes of the Andes below the most hostile ‘edge of life’ was also strong and multiple stress factors such as the mountainous oxygen regime, high UV radiation and lack of leaf detritus from montain forest were indicated as drivers.


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Summary

Mountain regions cover about 27% of the Earth’s surface, encompassing glacial stocks and freshwater reservoirs that play a valuable role providing ecosystem services to large human populations living downstream. Mountain regions are unique through the heterogeneity of ecosystems and diversity of climates arising from their sharp altitude gradients. When approaching the summits, these factors create exceptional harsh conditions for life, which may be challenged even further by man-made disturbances: life at the edge.

The aim of this thesis was to identify potential drivers of diversity in poorly studied benthic invertebrate assemblages in high altitude Andean streams and to elucidate the mechanisms that enable them to cope with ‘life at the edge’. To this purpose, the following objectives were set:

- To describe for the first time the benthic invertebrate assemblages in high altitude Andean streams and to relate their composition to the strong gradients in abiotic factors.
- To unravel the role of melanin as a strategy against harmful UV-B radiation and metal exposure.
- To study the genetic diversity of benthic invertebrates occurring under extreme conditions and showing specific defense strategies.

High altitude creates unique challenging conditions to biota that limit the diversity of benthic communities. Because environmental pollution may add further stress to life at high altitude, in chapter 2 I first explored the effect of metal pollution on macroinvertebrate community composition in Andean streams between 3500 and 4500 m above sea level (a.s.l.) during wet and dry seasons. At polluted sites, showing a high conductivity and a low pH, metal concentrations ranged from 8-fold up to 3500-fold higher than at reference sites. The cumulative criterion unit allowed quantifying the potential toxicity of metal mixtures at the contaminated sites. Principal Component Analysis of physical chemical variables showed that reference sites were more likely to be structured by transparency, water discharge, and current velocity, while polluted sites appeared to be determined by metals and conductivity. Canonical Correspondence Analysis indicated a strong influence of highly correlated metals in structuring invertebrate communities, which were dominated by dipterans, coleopterans, collembolans, and mites at polluted sites. At reference sites crustaceans, ephemeropterans, plecopterans, and trichopterans were the most representative taxa. It was concluded that severe metal pollution induced changes in macroinvertebrate community composition in high altitude Andean streams, with a replacement of sensitive taxa by more tolerant taxa. Yet, relatively species-rich communities persisted under harsh conditions.
In chapter 3 I evaluated the differential effects of metal pollution and altitude on benthic macroinvertebrate community composition. Polluted sites were characterized by high metal concentrations and low pH, and high altitude sites by high ultraviolet-B (UV-B) radiation and low concentrations of dissolved organic matter. Canonical Correspondence Analysis indicated that the patterns in faunal composition were best explained by metal pollution followed by altitude, with dipterans and collembolans occurring mostly under harsh conditions of high altitude and high pollution levels. It was concluded that in highland Andean streams metal leaching from igneous rock and altitude may be important factors modulating benthic macroinvertebrate communities reducing their numbers and changing their composition towards specialized taxa.

High metal concentrations and an elevated UV-B radiation in high altitude Andean streams create highly selective conditions for life, allowing the persistence of only a few specialized taxa, including chironomids. Therefore, the aim of chapter 4 was 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 2-fold higher at 4000 m compared to 3000 m, and 2-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 was 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.

Photoprotective pigments in benthic macroinvertebrates may reduce the damage caused by the blistering UV-B radiation in Andean high altitude streams above 3500 m. Therefore, the aim of chapter 5 was 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. I 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 two-fold that at 3200 m, four-fold that at 1900 m, and five-
fold that at 650 m. The melanin concentration in families belonging to Ephemeroptera, Trichoptera, Diptera and Turbellaria was two-fold higher at 4000 m compared to 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 arose 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 two-fold higher at 1900 m compared to 650 m. In *Baetodes* melanin at 4000 m was two-fold that at 650 and 1900 m, while in *Thraulodes* it was almost three-fold higher at 4000 m compared to 3200 m. In *Tupiara*, the differences in melanin levels were likely associated to species with different vertical distribution, while in *Dactylobaetis* these differences were interpreted as phenotypic plasticity. These 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.

As described above, chironomids are among the few dominant insect taxa present under the harshest environmental conditions in polluted high altitude Andean streams. Yet, the question remained if the dominance of chironomids was due to either an adaptive capacity of few species (population differentiation) or to a diversity of species with different capacities to cope with environmental extremes (species composition). To answer this question, in chapter 6 the genetic composition of the chironomid communities from reference and metal polluted streams at 3000 and 4000 m a.s.l. was determined by mitochondrial cytochrome oxidase I (COI) gene sequencing and construction of a phylogenetic tree. At 3000 m the reference site was inhabited by 6 phylogenetic species, completely different from the 3 present at 4000 m, indicating a strong sorting of species according to altitude. Only one phylogenetic species was present at the metal-rich sites. This metal tolerant species was the same at 3000 and 4000 m, and unique for the polluted sites, indicating that the extreme selection pressure by metal exposure overruled altitude driven selection. It was concluded that altitude imposes strict limits to the distribution of chironomid taxa creating a strong vertical zonation on the slopes of the high Andes, yet, selection in acidic metal-rich waters is so strong that altitude driven selection is overruled, leading to predominance of a unique metal tolerant taxon.

In conclusion, high altitude Andean streams harbor a quite diverse benthic community represented by the major groups of invertebrates. Evidence is provided that abundant insect species have not been described taxonomically and that unique genotypes occur, probably as a result of the geological history of the Andes and the strong selection
for high altitude tolerance. Cuticular pigmentation of larvae was demonstrated to form an inducible sunscreen against scorching UV radiation, but the pigment melanin was also effective in mitigating metal stress. Thus a single trait of the fauna enabled species to cope with the combined stressor UV radiation and metal exposure from leaching rock. The only species from the stress tolerant chironomids that survived the most harsh condition of a metal-rich, UV blasted site was a non-identifiable chironomid, characterized as a new haplotype. The vertical zonation of insect fauna on the slopes of the Andes is strong and I identified multiple stress factors such as the mountainous oxygen regime, high UV radiation and metal leaching as drivers of the composition of benthic assemblages in high altitude Andean streams. It is concluded that selection is strong at all altitudes in the Andes, but is most evident at high altitude and high metal exposure: at ‘the edges of life’.
Samenvatting

Gebergtes bedekken ongeveer 27% van het aardoppervlak en bevatten gletsjers en zoetwatervoorraden die waardevolle ecosysteemdienssten verzorgen voor grote hoeveelheden mensen benedenstrooms. Gebergtes zijn uniek vanwege de heterogeniteit aan ecosystemen en de diverse klimatologische omstandigheden, veroorzaakt door de steile hoogtegradiënten. Richting de toppen worden de randvoorwaarden voor het leven uitzonderlijk zwaar, wat nog verergerd kan worden door menselijke verstoringen: leven op de rand.

Het doel van dit proefschrift was het identificeren van de factoren die de diversiteit van de slecht onderzochte levensgemeenschappen van benthische ongewervelden in hooggelegen Andes rivieren sturen en de mechanismen op te helderen die deze organismen in staat stellen om te gaan met ‘leven op de rand’. Hiertoe zijn de volgende doelen gesteld:

- Het voor het eerst beschrijven van de levensgemeenschappen van benthische ongewervelden in hooggelegen Andes rivieren en hun soortsamenstelling relateren aan de sterke abiotische gradiënten.
- Het ophelderen van de rol van melanine als verdedigingsstrategie tegen schadelijke UV-B straling en blootstelling aan metal.
- Het onderzoeken van de genetische diversiteit van benthische ongewervelden die voorkomen onder deze extreme omstandigheden en die specifieke verdedigingsstrategiën vertonen.

Grote hoogte veroorzaakt unieke uitdagingen voor organismen die de diversiteit van benthische levensgemeenschappen beperken. Omdat milieuverontreiniging nog meer stress toevoegt aan het leven op grote hoogte heb ik in hoofdstuk 2 eerst het effect van metaalverontreiniging op de samenstelling van de levensgemeenschappen van benthische ongewervelden in Andes rivieren tussen de 3500 en 4500 m hoogte onderzocht gedurende het natte en droge seizoen. In verontreinigde rivieren, die een hoge geleidbaarheid en een lage pH vertoonden, varieerden de metaalconcentraties van 8 tot 3500 keer hoger dan die in de referentie rivieren. PCA analyse van de fysisch-chemische factoren toonden aan dat de referentie locaties vooral werden gegroepeerd op basis van doorzicht, waterafvoer en stroomsnelheid, terwijl de verontreinigde rivieren vooral bepaald werden door metalen en geleidbaarheid. CCA analyse wees op een sterke invloed van de sterk positief gecorreleerde metaalconcentraties op samenstelling van de levensgemeenschappen van benthische ongewervelden, die in verontreinigde rivieren werden gedomineerd door vliegen en muggen, waterkevers, springstaarten en watermijten. In de referentierivieren waren kreeftachtigen, haften, steenvliegen en kokerjuffers de meest voorkomende taxa. Geconcludeerd werd dat de ernstige metaalverontreiniging veranderingen veroorzaakte in de samenstelling van de levensgemeenschappen van benthische ongewervelden in
hooggelegen Andes rivieren, waarbij gevoelige taxa vervangen werden door meer tolerante taxa. Daarnaast kwamen relatief soortenrijke levensgemeenschappen voor onder extreme omstandigheden.

In hoofdstuk 3 heb ik de uiteenlopende effecten van metaalverontreiniging en hoogte op de samenstelling van de levensgemeenschappen van benthische ongewervelden in Andes rivieren onderzocht. De verontreinigde rivieren werden gekenmerkt door hoge metaalconcentraties en een lage pH en de hoogstgelegen rivieren door hoge UV-B straling en lage concentraties opgelost organisch materiaal. CCA-analyse toonde aan dat de patronen in fauna samenstelling het best werden verklaard door metaalverontreiniging gevolgd door hoogte, waarbij vooral vliegen, muggen en springstaarten voorkwamen onder de meest extreme omstandigheden, grote hoogte en metaalverontreiniging. Geconcludeerd werd dat in hooggelegen Andes rivieren metaaluitspoeling uit ertsrijk gesteente en hoogte belangrijke factoren zijn die levensgemeenschappen van benthische ongewervelden beïnvloedden, leidend tot verminderde aantallen en een verschuiving in soortsamenstelling in de richting van gespecialiseerde taxa.

Hoge metaalconcentraties en UV-B straling in hooggelegen Andes rivieren creeren strenge randvoorwaarden voor het leven, waardoor hier slechts een beperkt aantal taxa kan voortbestaan, waaronder dansmuggen. Daarom was het doel van hoofdstuk 4 om de mechanismen te bepalen die het deze dansmuggen mogelijk maken om voort te bestaan onder deze stressvolle condities. De hypothese was dat melanine zowel de negatieve effecten van UV-B straling als van metalen te niet zou kunnen doen. Melaninegehaltes werden bepaald in dansmuggen uit schone en metaalverontreinigde rivieren op 3000 en 4000 meter hoogte. Deze waren op 4000 meter twee maal hoger dan op 3000m en in verontreinigde rivieren twee maal hoger dan in referentie rivieren, op beide hoogtes. Deze veldwaarnemingen werden experimenteel bevestigd door het vaststellen van de gecombineerde effecten van koper en UV-B straling op overleving en melanine concentratie in larven van de model dansmugsoort Chironomus riparius. In de laboratoriumexperimenten werden de hoogste melanineconcentraties aangetroffen in larven die toxische koperconcentraties overleefden, maar niet in larven die werden blootgesteld aan UV-B straling. Voorbehandeling met UV-B straling vermindere de gevoeligheid van de larven voor UV-B straling en voor de behandeling koper plus UV-B straling. Geconcludeerd werd dat in het veld melanine dansmuggen gedeeltelijk beschermd tegen zowel verhoogde metaalconcentraties als UV-B straling, wat hen de mogelijkheid biedt om voort te bestaan onder de meest extreme condities in rivieren op grote hoogte.

Beschermende pigmenten in benthische ongewervelden zouden de schade door de sterke UV-B straling in Andes rivieren op grote hoogte boven 3500 m kunnen verminderen. Daarom was het doel van hoofdstuk 5 om vast te stellen of melanisering van benthische ongewervelden in Andes rivieren op grote hoogte een adaptieve respons is op hoge Uv-B
straling. Om te onderzoeken of hoogte gerelateerde verschillen in melanine concentraties tussen taxa werden veroorzaakt door veranderingen in soortencomposities of door populatiedifferentiatie zijn een aantal haftensoorten genetisch geïdentificeerd. UV-B straling werd gemeten van 650 tot 4000 m hoogte en de melanineconcentraties in diverse ordes van benthische ongewervelden ver zameld op deze hoogtes werd vergeleken. Vijf genera van de haftenfamilie Baetidae werden genetisch geïdentificeerd tot op soorteniveau. DNA sequencing werd uitgevoerd op individuele larvale poten om genetisch identieke individuen samen te kunnen nemen ten behoeve van de melanine analyse in de bijbehorende lichamen. De UV-B straling op 4000 m hoogte was twee maal hoger dan op 3200 meter, vier maal hoger dan op 1900 m en vijf maal hoger dan op 650 m. De melanine concentratie in families behorende tot de haften, kokerjuffers, vliegen en muggen en platwormen was twee maal hoger op 4000 m dan op 3200 m, maar verschillen niet tussen taxa en tussen seizoenen. Vijf genera van de haftenfamilie Baetidae werden geïdentificeerd: Americabaetis, Dactylobaetis, Tupiara, Baetodes en Thraulodes. Er bleken genetische verschillen te zijn tussen de Americabaetis soorten van 4000 m in de Cordillera Blanca en van 3200 m uit het Rímac vallei en tussen Tupiara taxa van 650 en 1900 m uit de Rímac vallei. In Americabaetis was de melanineconcentratie vijf maal hoger op 4000 m dan op 1900 m en in Dactylobaetis en Tupiara het twee maal hoger op 1900 m dan op 650 m. In Baetodes was de melanineconcentratie op 4000 m twee maal hoger dan op 650 en 1900 m en in Thraulodes was het bijna drie maal hoger op 4000 m dan op 3200 m. Wat betreft het geslacht Tupiara waren de verschillen in melanineconcentraties gerelateerd aan soorten met een verschillende verticale distributie, terwijl voor het geslacht Dactylbaetis deze verschillen werden geïnterpreteerd als fenotypische plasticiteit. Deze bevindingen toonden dus aan dat haftensoorten binnen één familie zowel constitutieve als aanpasbare melanineconcentraties kunnen bevatten, wat hen de mogelijkheid biedt om te gaan met de sterk selectieve UV-B straling. Aanpasbare melanineconcentraties zijn algemeen waargenomen onder gematigde UV-B straling regimes, maar de constitutieve hoge melaninconcentraties zijn waarschijnlijk een eigenschap van ongewervelden in de hoge tropische Andes.

Zoals hierboven beschreven behoren de dansmuggen tot de Weinige dominante taxa die aanwezig zijn onder de meest extreme condities in metaalverontreinigde hooggelegen rivieren in de Andes. De vraag bleef echter of de dominantie van de dansmuggen veroorzaakt werd door adaptatie binnen enkele soorten (populatiedifferentiatie) of door diverse soorten met verschillende capaciteiten om om te gaan met extreme milieumogelijkheden. Om deze vraag te beantwoorden is in hoofdstuk 6 de genetische samenstelling van dansmuggenlevensgemeenschappen van referentie en metaal verontreinigde rivieren op 3000 en 4000 m onderzocht met behulp van mitochondriale cytochroom oxidase I (COI) gen sequentie en het construeren van een fylogenetische boom. Op 3000 m werd de referentie rivier bewoond door zes fylogenetische soorten die volledig
verschillen van de drie soorten aanwezig op 4000 m, wat duidde op een sterke verdeling van soorten over hoogte. In de metaalverontreinigde rivieren werd slechts één fylogenetische soort aangetroffen. Deze metaaltolerante soort was zowel aanwezig op 3000 en 4000 m en was uniek voor de verontreinigde rivieren, wat aantoonde dat extreme selectiedruk door metaalverontreiniging de selectiedruk door hoogte overheerste. Geconcludeerd werd dat hoogte sterke beperkingen oplegde aan de verspreiding van dansmuggensoorten, leidend tot een sterke verticale verdeling van soorten over de hoogtes in de Andes, maar dat selectie in zure metaalverontreinigde rivieren zo sterk is dat deze de selectie door hoogte overheerst, leidend tot het voorkomen van een uniek metaal tolerant taxon.

Concluderend werd dat rivieren op grote hoogte in de Andes een behoorlijk diverse levensgemeenschap van benthische ongewervelden herbergen, vertegenwoordigd door de belangrijkste groepen ongewervelden. Tevens is het bewijs geleverd dat algemeen voorkomende insectensoorten taxonomisch nog niet beschreven waren en dat unieke fylogenetische soorten voorkomen, hoogstwaarschijnlijk ten gevolge van de geologische geschiedenis van de Andes en de sterke selectie voor tolerantie tegen hoogte. Er werd aangetoond dat larvale pigmentatie een induceerbaar ‘zonnescherm’vormt wat bescherming biedt tegen de sterke UV-B straling, maar het pigment melanine bleek ook effectief metaal stress tegen te gaan. Een enkele eigenschap van de fauna stelden soorten dus in staat om te gaan met de gecombineerde stress van UV-B straling en blootstelling aan metalen die uitspoelen uit metaalrijke erts. De enige soort van de stress tolerante dansmuggen die voorkwam onder de meest extreme omstandigheden, de hoogste UV-B rijke metaalverontreinigde rivier, was een ongeïdentificeerde dansmug, gekenmerkt als een nieuwe fylogenetische soort. De verticale verdeling van de insectenfauna over de hoogtes van de Andes is sterk en ik identificeerde meerdere stress factoren, zoals de montane zuurstofhuishouding, sterke UV-B straling en uitspoeling van hoge metaalconcentraties als factoren die de samenstelling van de levensgemeenschappen van benthische ongewervelden sturen. Geconcludeerd werd dat er een sterke selectie optreedt op alle hoogtes in de Andes, maar dat deze het meest uitgesproken is onder blootstelling aan grote hoogte en hoge metaalconcentraties: het leven op de rand.
Las regiones montañosas cubren alrededor del 27% de la superficie de la Tierra y abarcan valiosas reservas glaciares y de agua dulce que tienen una importante función como proveedoras de servicios ecosistémicos para las poblaciones humanas. Estas regiones son únicas por la heterogeneidad de sus ecosistemas y la diversidad de sus climas, los cuales emergen del pronunciado gradiente altitudinal. Al acercarse a las cumbres, estos factores crean condiciones de supervivencia excepcionalmente difíciles, las cuales pueden acentuarse por alteraciones de origen humano, dando lugar a los llamados ‘límites de la vida’.

El propósito de esta tesis fue identificar potenciales moduladores de la diversidad de las comunidades de invertebrados bentónicos en ríos altoandinos, poco estudiadas hasta el momento, y elucidar los mecanismos que les permiten enfrentar estos ‘límites de la vida’. Para ello, se establecieron los siguientes objetivos:

- Describir por primera vez las comunidades de invertebrados bentónicos en ríos altoandinos y relacionar su composición con un gradiente de factores abióticos.
- Elucidar la función de la melanina como una estrategia de protección contra la nociva exposición a la radiación ultravioleta B (UV-B) y metales.
- Estudiar la diversidad genética de los invertebrados bentónicos que muestran estrategias de defensa específicas bajo condiciones ambientales extremas.

La altitud crea condiciones que desafían la vida y limitan la diversidad de las comunidades bentónicas. Debido a que la contaminación ambiental puede añadir aun mayor estrés para la vida a gran altitud, en el capítulo 2 se exploró el efecto de los metales en la composición de las comunidades de macroinvertebrados en arroyos altoandinos entre 3500 y 4500 m sobre el nivel del mar (s.n.m.) durante las temporadas húmeda y seca. En los sitios contaminados, que mostraron una elevada conductividad y bajo pH, la concentración de metales fue entre 8 y 3500 veces más que en los de referencia. La unidad de criterio acumulado (CCU) permitió cuantificar la potencial toxicidad de la mezcla de metales en los lugares contaminados. El Análisis de Componentes Principales de las variables físicas y químicas mostró que los sitios de referencia estuvieron determinados por la transparencia, caudal y velocidad de corriente, mientras que los contaminados lo estuvieron por los metales y la conductividad. El Análisis de Correspondencia Canónica indicó una fuerte influencia de metales altamente correlacionados en la estructura de las comunidades de invertebrados, dominadas por dípteros, coleópteros, colémbolos y ácaros en los sitios contaminados. En los sitios de referencia, los crustáceos, efemerópteros, plecópteros y tricópteros fueron los grupos más representativos. Se concluyó que la severa contaminación por metales produjo cambios en la composición de la comunidad de macroinvertebrados en arroyos altoandinos, con un reemplazo de taxa sensibles por otros más tolerantes. Sin
Resumen

embargo, aun bajo las condiciones más severas, se encontró una comunidad relativamente diversa.

En el capítulo 3 se evaluó el efecto diferencial de la contaminación por metales y la altitud en la composición de las comunidades de macroinvertebrados bentónicos. Los sitios contaminados estuvieron caracterizados por altos niveles de metales y un bajo pH, y los sitios más elevados por una intensa radiación UV-B y bajas concentraciones de materia orgánica disuelta. El Análisis de Correspondencia Canónica indicó que los patrones de la composición de la macrofauna fueron mejor definidos por los metales, seguidos por la altitud, siendo los dípteros y colémbolos predominantes bajo las condiciones más severas de altitud y contaminación. Se concluyó que en arroyos altoandinos, la lixiviación natural de metales de roca y la altitud son importantes moduladores de las comunidades de macroinvertebrados bentónicos, reduciendo su número y cambiando su composición hacia grupos especializados.

Las elevadas concentraciones de metales y radiación UV-B crean condiciones altamente selectivas para la vida en arroyos altoandinos, permitiendo la presencia de solo unos cuantos grupos especializados, incluyendo los quironómidos. Por ello, el propósito del capítulo 4 fue determinar los mecanismos que sustentan la persistencia de los quironómidos bajo estas condiciones de estrés múltiple, teniendo como hipótesis que la melanina puede contrarrestar los efectos adversos tanto de la radiación solar como de los metales. Se determinó la melanina en quironómidos de arroyos de referencia y contaminados a 3000 y 4000 m de altitud, siendo a 4000 m el doble que a 3000 m, y el doble en arroyos contaminados que en los de referencia a ambas altitudes. Las observaciones de campo fueron verificadas experimentalmente evaluando los efectos combinados del Cu y UV-B en la supervivencia y concentración de melanina en larvas de la especie modelo *Chironomus riparius* (Chironomidae, Diptera). En el laboratorio, la mayor concentración de melanina se encontró en larvas que sobrevivieron a concentraciones tóxicas de Cu, pero no en aquellas expuestas a la más alta radiación UV-B. La pre-exposición a UV-B redujo la sensibilidad de las larvas a UV-B y a Cu+UV-B. Se concluyó que en el campo, la melanina puede proteger a los quironómidos contra elevadas concentraciones de metales y radiación solar, permitiéndoles persistir bajo las condiciones más severas en arroyos a gran altitud.

Los pigmentos fotoprotectores pueden reducir el daño causado por una intensa radiación UV-B en macroinvertebrados bentónicos de arroyos altoandinos ubicados por encima de los 3500 m. Por ello, el objetivo del capítulo 5 fue determinar si la melanización en macroinvertebrados de estos arroyos es una respuesta adaptativa a la elevada radiación UV-B. Para explorar si las diferencias en la concentración de melanina entre taxa, relacionadas con la altitud, se debieron a variaciones en la composición de las comunidades o a la diferenciación de poblaciones, las especies de efemerópteros fueron identificadas genéticamente. Se determinó la radiación UV-B desde los 650 m hasta los 4000 m y se
comparó la concentración de melanina de distintos órdenes de macroinvertebrados bentónicos en estas altitudes. Cinco géneros pertenecientes a efímeras de la familia Baetidae fueron identificados genéticamente a nivel de especie. Se llevó a cabo el secuenciamiento de ADN en las patas de larvas individuales para agrupar individuos genéticamente similares antes del análisis de pigmentos en los correspondientes cuerpos. La radiación UV-B a 4000 m fue el doble que a 3200 m, cuatro veces mayor que a 1900 m, y cinco veces más que a 650 m. La concentración de melanina en las familias pertenecientes a Ephemeroptera, Trichoptera, Diptera y Turbellaria fue dos veces mayor a 4000 m que a 3200 m, pero no fue distinta entre taxa o entre temporadas. Se identificaron cinco géneros en la familia Baetidae: *Americabaetis, Dactylobaetis, Tüpiara, Baetodes y Thraulodes*. Hubieron diferencias genéticas entre *Americabaetis* sp. a 4000 m en la Cordillera Blanca y a 3200 m en el valle del Río Rímac, y entre *Tüpiara* a 650 y 1900 m en éste último. En *Americabaetis* la melanina aumentó cinco veces de 1900 a 4000 m, mientras que en *Dactylobaetis* y *Tüpiara* fue el doble a 1900 m que a 650 m. En *Baetodes* la melanina fue el doble a 4000 m que a 650 y 1900 m, mientras que en *Thraulodes* fue casi el triple a 4000 m que a 3200 m. En *Tüpiara*, las diferencias en los niveles de melanina estuvieron probablemente asociados a especies con diferente distribución vertical, mientras que en *Dactylobaetis* estas diferencias fueron interpretadas como plasticidad fenotípica. Estos resultados indican que las especies de efímeras pertenecientes a la misma familia tienen concentraciones tanto constitutivas como regulables de melanina, permitiéndoles hacer frente a ambientes con niveles de UV-B altamente selectivos. La regulación de melanina ha sido observada bajo condiciones moderadas de UV-B, mientras que su elevada concentración constitutiva sea probablemente un atributo de los invertebrados de los trópicos a gran altitud.

Como se ha descrito arriba, los quironómidos se encuentran entre los escasos grupos dominantes de insectos bajo las condiciones ambientales más severas en arroyos altoandinos contaminados. Sin embargo, persiste la pregunta si la dominancia de los quironómidos se debe a la capacidad adaptativa de unas pocas especies (diferenciación de poblaciones) o a una diversidad de especies con diferentes capacidades para hacer frente a ambientes extremos (composición de especies). Para responder esta pregunta, en el capítulo 6 la composición genética de las comunidades de quironómidos de arroyos de referencia y contaminados por metales a 3000 y 4000 m fue determinada mediante el secuenciamiento del gen de la citocromo oxidasa I mitocondrial (COI) y la construcción de un árbol filogenético. El sitio de referencia a 3000 m estuvo habitado por seis especies filogenéticas completamente diferentes de las tres presentes a 4000 m, indicando una fuerte selección de las especies por la altitud. En los lugares contaminados solo se encontró una especie tolerante a los metales. Esta especie, única para los sitios contaminados, estuvo presente a 3000 y 4000 m, indicando que la extrema presión de selección de los metales predominó sobre la altitud. Se concluyó que la altitud crea límites estrictos para la distribución de
quironómidos y una fuerte zonificación vertical en los Andes. Sin embargo, la selección en aguas ácidas contaminadas con metales es mayor, y conlleva a la predominancia de un único taxón tolerante a los metales.

Los arroyos altoandinos albergan una diversa comunidad bentónica representada por los mayores grupos de invertebrados. Se ha proporcionado evidencia que muchas especies de insectos no han sido aún descritas taxonómicamente, y que existen genotipos únicos, probablemente como resultado de la historia geológica de los Andes y de la fuerte selección para tolerar las grandes altitudes. Se demostró que la pigmentación cuticular de las larvas es una protección inducible contra una elevada radiación UV, y que la melanina es efectiva para mitigar el estrés producido por los metales. Así, un único atributo permitiría a estas especies hacer frente a la exposición combinada a la radiación UV y metales lixiviados de roca. La única especie entre los quironómidos tolerantes al estrés, que sobrevivió las condiciones más severas de radiación y contaminación, fue una no identificada, que se caracterizó como un nuevo haplotipo. Además de la fuerte zonificación vertical de la fauna de insectos en los Andes, se identificaron múltiples factores de estrés, como los regímenes de oxígeno propio de las montañas, la elevada radiación UV-B y la lixiviación de metales como moduladores de la composición de las comunidades bentónicas en arroyos altoandinos. Finalmente, existe una fuerte selección en todos los niveles altitudinales en los Andes, la cual es más evidente en las partes más altas y más contaminadas, esto es, en los ‘límites de la vida’.
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Curriculum vitae

Raúl Loayza Muro was born on March 7th 1975 in Lima, Peru. He spent his childhood in Lima and Palma de Mallorca, Spain. After returning to Peru, he followed lower school at Markham College and then at Winnetka School. Since he was eleven, he lived in Chaclacayo, a town in the country side of Lima, gifted with quietness, hills and sun all year round. The daily view of the lower Andes and clear skies from home arose his interest for nature. Some years later he obtained a Bachelor Degree in Sciences, Major in Biology at the Universidad Peruana Cayetano Heredia (UPCH) in Lima. He made his undergraduate thesis at the Department of Biochemistry and at the Institute of Tropical Medicine ‘Alexander von Humboldt’ (UPCH). Between January 1999 and May 2000 he spent a research period at the Laboratory of Chemical Ecology at the Universidad de Chile exploring the role of antioxidant and detoxifying enzymes on the tolerance of aphids to cereal secondary metabolites. After returning to Lima, he continued working at the Laboratory of Molecular Biology and Parasitology, but seeking new horizons and trying to pursue a new research line in environmental sciences, which at the moment was not available at UPCH. In 2002 he began his research in ecotoxicity of metals in Amazon freshwater mussels and soon after he expanded to benthic macroinvertebrates in high altitude metal-polluted streams in the Andes. By that moment he became an assistant professor and Head of the Laboratory of Ecotoxicology at the Faculty of Sciences and Philosophy. He did his PhD at the University of Amsterdam between 2009 and 2013. At present, he continues doing research at his laboratory at UPCH exploring the effects of climate change and glacial retreat on the acidification of headwaters and metal mobilization in Andean streams.