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

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**Publication date**  
2013

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#### **Citation for published version (APA):**

Loayza-Muro, R. A. (2013). *Life at the edge: Benthic invertebrates in high altitude Andean streams*.

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## ***Chapter 6***

### Metals and altitude drive genetic diversity of chironomids in Andean streams

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## **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 reference 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 (Villafaña 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.

## Materials and methods

### Study sites

Four sites in the Quillcayhuanca 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.

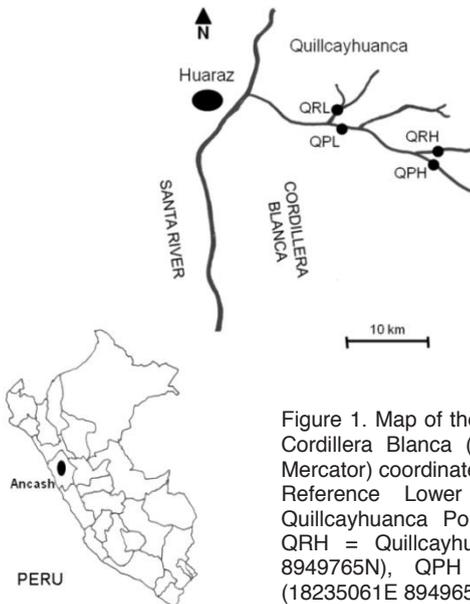


Figure 1. Map of the study area, indicating the sampling sites in the Cordillera Blanca (Peru). Labels and UTM (Universal Tranverse Mercator) coordinates for sites are as follows: QRL = Quillcayhuanca Reference Lower altitude (18224401E 8946087N), QPL = Quillcayhuanca Polluted Lower altitude (18224401E 8946087N), QRH = Quillcayhuanca Reference Higher altitude (18235408E 8949765N), QPH = Quillcayhuanca Polluted Higher altitude (18235061E 8949655N).

### 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

site with 1-L polypropylene bottles, preserved with 10 N HNO<sub>3</sub> 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 T<sub>10</sub>E<sub>1</sub> 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 H<sub>2</sub>O, 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 H<sub>2</sub>O 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 GenBank™ 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.

Altitude	Status	UV-B (W/m <sup>2</sup> )	Conductivity (μS/cm)	pH	Temperature (°C)	Oxygen (mg/L)
3000 m	Ref	2.31	73	8.0	15.0	6.1
	Pol	1.92	155	4.5	14.3	6.8
4000 m	Ref	3.06	50	7.0	8.2	6.0
	Pol	2.95	132	4.3	9.3	5.1

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.

Altitude	Status	Al	As	Ca	Co	Cu	Fe	Mn	Ni	Sr	Zn
3000 m	Ref	0.43	0.006	6.8	0.001	0.003	0.7	0.011	0.001	0.050	0.005
	Pol	1.66	0.009	12.1	0.014	0.082	2.3	0.561	0.026	0.088	0.243
4000 m	Ref	0.07	0.007	8.8	0.001	0.003	0.4	0.002	0.001	0.032	0.005
	Pol	1.69	0.011	15.7	0.020	0.087	3.2	0.644	0.022	0.077	0.211
Highest/lowest Metal conc.		24	2	2	20	29	16	322	26	4	49

### *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

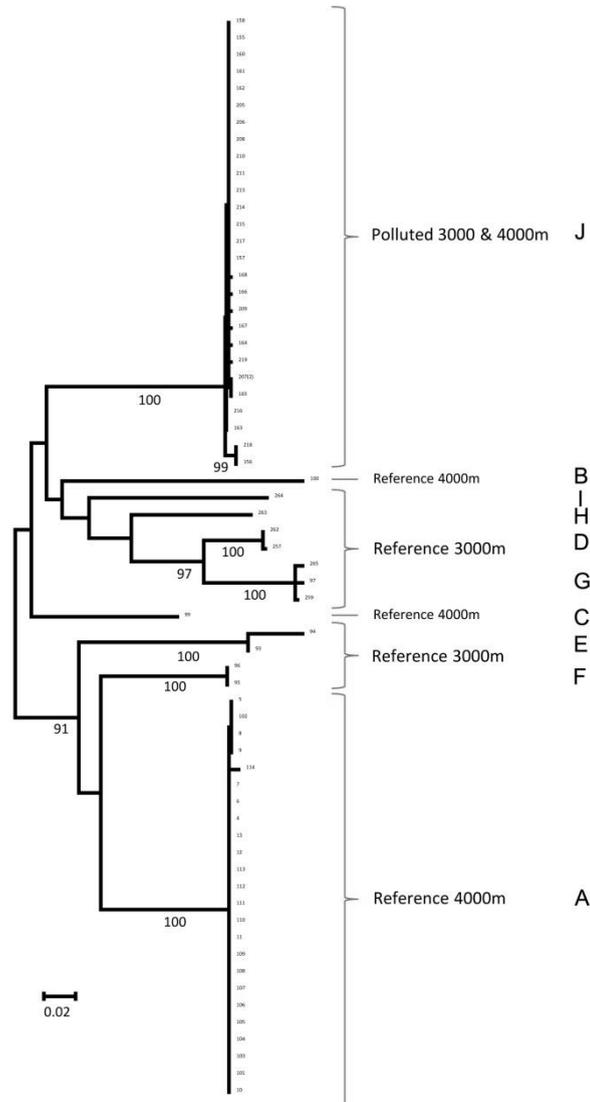


Figure 2. Phylogenetic relationships of chironomid taxa collected at reference and polluted sites at 3000 and 4000 m. The best fitting nucleotide substitution model used for inferring the maximum likelihood tree based on a 709 bp COI mtDNA sequence of 81 individuals was the GTR+G. The percentage of replicate 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.

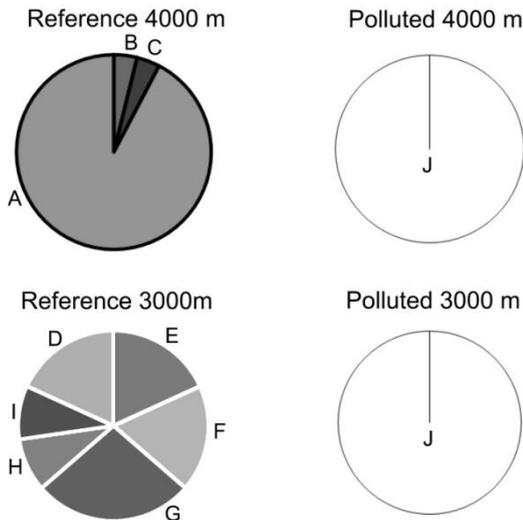


Figure 3. Relative abundance of chironomid phylogenetic species present at reference and polluted sites at 3000 and 4000 m. Letters A–C, D–I and J refer to different phylogenetic species obtained from Figure 2.

## 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.

*Acknowledgements.* This work was funded by the International Foundation for Science (Research Grant Agreement A/4426-2). We want to thank the assistance of Carlos Merino and Rolando Cruz for helping with field sampling. Raúl Loayza-Muro received a doctoral scholarship from the Program for Science and Technology of Peru (FINCyT, Contract No. 113-2009-FINCyT-BDE) for writing this paper at the University of Amsterdam.