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The altitudinal limit of *Leptohyphes* Eaton, 1882 and *Lachlania* Hagen, 1868 (Ephemeroptera: Leptohyphidae, Oligoneuriidae) in Ecuadorian Andes streams: searching for mechanisms

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ABSTRACT

We explored mechanisms determining the upper altitudinal limit of ephemeropterans from two different genera: *Leptohyphes* Eaton, 1882 (Leptohyphidae) and *Lachlania* Hagen, 1868 (Oligoneuriidae). For this, we (1) surveyed the two taxa in 165 stream sites along a wide altitudinal gradient; (2) sampled benthic fauna at short altitudinal intervals along a stream, from 2780 to 3150 m above sea level; (3) collected adults at the lowest and highest sites; and (4) transplanted nymphs from the lowest to the highest study site in our stream to determine survival over time. Densities of the two taxa declined gradually with altitude and both disappeared between 2950 and 3080 m a.s.l. The upper altitudinal limit in the stream seemed to be most closely related to mean oxygen saturation, temperature, and current velocity. Adults were collected where the nymphs were found, but not at the upstream site where the nymphs were absent, implying limited upstream dispersal of adults and some of the altitudinal constraint lying at the adult stage. Short-term survival of transplanted nymphs was lower than that of controls, suggesting that the distribution was limited at the juvenile stage, and that at least some of the altitudinal constraint is related to the abiotic stream environment.

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Introduction

The study of patterns in biological diversity along major geographical gradients is a topic of considerable interest in ecology (Lomolino 2001; Cadena et al. 2011; Kraft et al. 2011). The species richness of most groups of organisms decreases from lowlands to the highest altitudes, although not necessarily monotonically over the entire altitudinal range (Rahbek 2005; Leingärtner, Krauss, and Steffan-Dewenter 2014). Explaining these altitudinal patterns is a difficult, but essential task. The reasons for species disappearing along altitudinal

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gradients can be divided into (1) biotic interactions (competitive exclusion, predation), which may not be directly related to the physico-chemical environment, and/or (2) autecology and physiological tolerances, which should relate to some physico-chemical environmental variable(s).

Hitherto, most studies in both the terrestrial (e.g., Sanders 2002; Brehm, Homeier, and Fiedler 2003) and the aquatic environment (e.g., Suren 1994, Jacobsen 2004) have simply described altitudinal patterns in insect diversity and in some cases analysed the correlation with environmental factors (Werenkraut and Ruggiero 2014). However, environmental factors are often closely inter-related along altitudinal gradients, such as air temperature and area (Kluge, Kessler, and Dunn 2006), air temperature and precipitation (Brehm, Colwell, and Kluge 2007), and temperature and dissolved oxygen in stream water (Jacobsen 2008a). To proceed in our understanding of these diversity patterns, we need to pursue causal explanations by conducting experiments focusing on selected taxa around their altitudinal limit. If we can explain the disappearance of some taxa, then we should be much better able to propose mechanisms responsible for the observed general patterns.

The clear decrease in taxon richness of macroinvertebrates with increasing altitude in Ecuadorian streams has previously been described (Jacobsen 2003, 2004, 2008a). Here we report our attempt to gain more insight into the mechanisms determining specific distributional limits along an altitudinal gradient of ephemeropteran nymphs of two genera: *Leptohyphes* Eaton, 1882 (Leptohyphidae) and *Lachlania* Hagen, 1868 (Oligoneuriidae). The study consisted of four steps. (1) To determine altitudinal limits of these two taxa, we compiled data on their occurrence in a large number of stream sites covering a wide altitudinal gradient and geographic area. Good measurements of water temperature and oxygen saturation were available from 30 of these sites, and this allowed us to analyse occurrence in relation to these factors. (2) We collected quantitative samples of aquatic nymphs at short altitudinal intervals along a stream, from where these genera were abundant up to where they disappeared from the stream. Here we intended to determine how populations thin out towards higher altitudes and whether densities were related to environmental site characteristics. (3) We collected adults at the lowest and at the highest study sites of our stream. Our aim here was to determine whether the aquatic nymphs and their terrestrial adults had similar altitudinal limits. (4) Finally, we performed an experiment transplanting aquatic nymphs held in protected mesh bags from the lowest to the highest site to determine survival over time. If short-term mortality was higher for transplanted compared to control animals, this should indicate that at least some of the altitudinal constraint lies at the juvenile stage, and that this is related to the abiotic environment rather than to biotic interactions with predators or competitors.

Material and methods

Study organisms

We focused on ephemeropterans of two genera: *Leptohyphes* (Leptohyphidae) that have been classified as true scrapers and *Lachlania* (Oligoneuriidae) that are active filterers (Merritt and Cummins 1996; Baptista et al. 2006). Both taxa seem to have an upper altitudinal limit around 3000 m a.s.l. in Ecuadorian streams (Jacobsen 2004). Representatives of these two genera were selected because they were relatively easy to handle (as opposed

to most species of e.g., Baetidae), were present in the experimental stream in relatively high numbers (see Río Guapal section below), were easy to identify in the field (at least to genus), and have a quite similar upper altitudinal limit. There is very little information about Ephemeroptera in the high equatorial Andes and this often hinders reaching species level when identifying specimens. In the case of *Leptohyphes*, eight species have been described and recorded from Ecuador based on specimens from limited sites (Wang, Sites, and McCafferty 1998; Molineri 2003; Domínguez, Hubbard, Pescador, Molineri, and Nieto 2013). At least 10 more species have been recorded from Colombia, Brazil, and Peru (Domínguez et al. 2013). For our specimens, we followed the key in Molineri (2003) but were unable to reach the species level. In the case of *Lachlania*, two species have been recorded from Ecuador and three from Colombia, Brazil, and Peru (Domínguez et al. 2013). Nevertheless, we know of no keys available for South American *Lachlania* nymphs. For these reasons, we identified our specimens only to genus (Fernández and Domínguez 2001; Domínguez, Molineri, and Nieto 2009).

Large-scale distribution data

We compiled our biological data on fauna from 165 stream sites distributed along an altitudinal gradient from 100 to 4000 m a.s.l. in northern Ecuador. A large part of this data is published in Jacobsen (2004, 2008a). This allowed us to visualize the frequency of occurrence along a wide altitudinal gradient of *Leptohyphes* and *Lachlania*. Data on the occurrence of the two genera were combined with measurements of altitude, mean water temperature, and mean oxygen saturation from 30 stream sites between 2600 and 4000 m a.s.l. given in Jacobsen (2008a). These data were used to determine the lowest mean oxygen % and mean temperature, at which each of these taxa occur in nature, and to compare their actual altitudinal limit with the altitude where these oxygen and temperature conditions usually occur based on regressions of altitude versus oxygen saturation and temperature, respectively. Although data on oxygen and temperature were not available for the 165 stream sites, we believe that patterns in these variables along altitudinal gradients are probably consistent. Therefore, relating data of only 30 streams to the distribution of our study organisms along 165 stream sites is a valid approach.

The Río Guapal

The stream Río Guapal is part of the Río San Pedro-Río Pita catchment, originating high in the páramos and draining the inter-Andean valley south and east of Quito. This catchment has a mean annual rainfall of 1500 mm and is the upper part of the Río Guayllabamba basin, a major tributary to Río Esmeraldas emptying into the Pacific Ocean. Along the entire study reach, the stream was surrounded by a mixture of vegetation comprising grass, scrubs, and small trees, such as *Alnus acuminata* Kunth, 1817, *Oreopanax confusum* Marchal, 1879, *O. corazonensis* Harms, 1942 and *Polylepis reticulata* Hieron, 1896 mixed with cultivated land and pasture.

Site characteristics

All field work was done between January and August 2005. Five sampling sites between 2780 and 3150 m a.s.l. along a 5 km stretch in Río Guapal were selected with an altitudinal

difference of 70–130 m and a horizontal distance of 0.7–1.8 km between two neighbouring sites. The relatively narrow altitudinal range of the study was chosen to maximize data-density around the altitudinal limit of the two taxa, i.e., ca. 3000 m a.s.l. We believe that studying sites along the same stream reduces the undesired variability in environmental parameters (in particular, water chemistry) and the importance of these in structuring benthic macroinvertebrate communities. In addition, studying such a limited reach at a high Andean stream probably reduced the risk of working with more than one species from each genera.

At each site, we measured some basic environmental parameters that might influence the distribution of *Leptohyphes* and *Lachlania*. Mean water temperature was recorded using max–min thermometers (Ningbo, model HT-066) for periods from three to five days. A few spot measurements of dissolved oxygen (using an oxymeter YSI 58, YSI), conductivity, and pH (using a water quality monitor 600XL, Endeco/YSI) were made at each field visit. Mean current velocity (in $0.6 \times$ water depth) was measured with a digital flowmeter (Höntzsch HFA) with a vane wheel sensor at 4–8 points along 3 transects at each site. Recordings of water depth and bottom substratum type were made along these same transects.

We estimated area specific benthic periphython biomass by using chlorophyll *a* (including *pheopigments*) as an estimator (Københavns Universitet 1973). At each stream site, 8 cobble stones were randomly collected and placed for 24 h in 96% ethanol. The absorbance at 665 and 750 nm was measured with a spectrophotometer (Termo Spectronic Helios Beta). The area *A* (in m²) of each stone was estimated using the formula proposed by Dall (1979): $A = 1.2 \times (L \times W \times H)$, where *L*, *H*, and *W* are the mean length, height, and width of the stone (in m), respectively.

To measure the amount of suspended particles, important for filter feeders like *Lachlania*, we filtered known volumes of stream water through glass-fibre filters (Whatman GF/C). The filters had been precombusted in a muffle furnace at 500 °C for 2 h. After filtration, filters were dried at 105 °C and weighed using a SARTORIUS CP124S electrobalance. They were once again combusted at 500 °C for 1 h and weighed. The amount of suspended organic matter in the known amount of stream water was measured as the difference between initial and final weight (i.e., weight of dry filter minus weight of combusted filter). This procedure was done twice for each stream.

Benthic sampling

Surber sampling was used to assess density of benthic macrofauna and organic matter along the stream. At each site, six quantitative Surber samples (area: 20 cm × 25 cm, mesh size: 200 μm) were randomly collected in a standardised gravel–pebble–cobble substratum in riffle-run habitats. We also standardised the sampling time by disturbing the substratum for two minutes for each surber sample. We collected a total of 30 benthic samples (6 surbers × 5 sites) that were labelled and preserved in 75% ethanol and transported to the laboratory where they were rinsed through a 200 μm sieve.

Benthic coarse particulate organic matter (CPOM) was obtained by filtering the remaining material from Surber samples through a 1 mm sieve. CPOM was oven dried (80 °C, 24 h), weighed, burnt in a muffle furnace (for 4 h at 550 °C) and re-weighed to calculate ash-free dry mass which was calculated as the difference between the initial and

final weight (dried CPOM minus combusted CPOM). Benthic fine particulate organic matter (FPOM) was the fraction that was held back by a 200 μm sieve after the first sieving through the 1 mm sieve.

Trapping of adults

At the two extreme sites (2780 and 3150 m a.s.l.), adults were sampled with 4 collection methods: malaise trap, light trap, sticky traps, and hand net. One malaise trap (height of 170 cm at the front part, 95 cm height at the back, 165 cm long, and 110 cm wide) was placed at each site at a distance of 1 m from the stream bank for 22 h. The light trap, consisting of a black light bulb of 15 W (Bioquip, Rancho Dominguez, USA) connected to a 12 V-battery, was located at 50 cm distance from the stream bank and lighted for 3 h at dusk (from 06:30 to 09:30 pm) and 3 h at dawn (from 03:00 to 06:00 am) for 1 day at each site. Three white trays filled with water and detergent were placed over a white sheet located under the black light. Four replicate sticky traps (20 cm \times 30 cm) were made using a transparent acetate sheet covered by a sticky solution of flaxseed oil placed from 50 cm to 1 m above the stream and attached to the bank vegetation stretched horizontally over the stream flyway, for a period of 5 days. Hand net sampling was done for 2 h at each site by sweeping the riparian vegetation and collecting adults that were seen flying close to the stream. All collections of adults were performed in dry weather, and all insects were preserved in 75% of ethanol and brought to the laboratory.

Transfer experiment

In order to have a more clear understanding about differences in oxygen (concentration and saturation) and temperature between the lower and upper sites, we placed oxygen probes (Oxylog, OxyGuard) at both sites that were programmed to record data every 30 minutes for 13 days, between April and May, 2005. Subsequently, in June 2005, 40 individuals of each of the 2 genera were collected by hand netting at the low elevation site (2780 m a.s.l.). For each taxon, 5 individuals were placed in 8 replicate rectangular bags (23 cm \times 13 cm \times 2 cm) made with nylon net (0.5 mm of mesh size). A standardized substratum composed by organic detritus and gravel was placed in each bag to provide a suitable environment for larvae and food source for *Leptohyphes*. *Lachlania* were assumed to feed from suspended particles in the inflowing water. For each taxon, 4 bags were transferred to 3150 m a.s.l., while the 4 others were given the same transport treatment but returned to the site of collection as controls. At both sites, mesh bags were placed in stream sections with swift current, in order to reduce the effect of large differences in current velocity between the two stream sites. The bags were examined every three days over nine days to assess nymph mortality. Because invertebrate sensitivity to environmental changes can be related to development stage, we also measured the cephalic width of all nymphs at the end of each exposure period. Cephalic width is a good indicator of nymph development for many insects (Rodríguez, Valdez, Vera, and Castillo 2000; Hernández-Martínez and Martínez 2003). The measurement was made to the nearest 0.01 mm using a graduated micrometric ocular stereomicroscope (Leica MZ 10, Leica Microsystems, Wetzlar, Germany).

Data analyses

As a control of the specific patterns observed for the two mayflies, the entire benthic fauna was described by three parameters. (1) Density (ind. m^{-2}), (2) total number of taxa collected in the Surber samples, and (3) a finite, individual-based rarefied richness (without replacement on 914 individuals, which was the lowest number in any of the 6 pooled Surber samples). The rarefied richness was calculated using the Species Diversity & Richness, version 3[®] software by Pisces Conservation Ltd and included to correct for the density effect of different number of individuals in samples on the number of taxa recorded. Relationships between faunal parameters and environmental site characteristics were tested with the Pearson product-moment correlations analysis. Due to the low number of datapoints ($N = 5$), significant correlations were difficult to obtain. Despite repeated correlation analyses to independent factors, the Bonferroni correction was not applied. In these analyses, the density of the total benthic fauna as well as that of *Leptohyphes* and *Lachlania* specimens was $\text{Log}(X+1)$ transformed.

In the transfer experiment, repeated measure data of nymph survival through time did not meet the assumption of independence. Consequently, to test whether temporal trends differed between the control and exposed groups, we fitted a linear regression line to the repeated measure data and used the slopes of the lines as response variables (Gotelli and Ellison 2013). The slopes of each of the four independent replicate groups were then compared using a two-way analysis of variance (ANOVA) to test for the significant effect of treatment (expose vs. control), taxa, and their interaction. In case of significance, we further performed a Tukey's *post hoc* analysis to compare survival rates between treatments at a given date. To detect the potential influence of cephalic width on nymph survival, we also performed an analysis of covariance (ANCOVA) with cephalic width as the covariate. All analyses were performed using R (R Development Core Team 2010).

Results

Large-scale distribution

According to our results, *Leptohyphes* is widespread and very common in streams in Ecuador, from sea level and up to 2800–3200 m a.s.l. (Figure 1). *Lachlania* is less common, does not occur at the lowest altitudes and has a peak in frequency of occurrence within the interval 2000–2400 m a.s.l. It also disappears between 2800 and 3200 m a.s.l. (Figure 1).

Mean water temperature and oxygen saturation in the 30 stream sites included in Jacobsen (2008a) and the occurrence of *Leptohyphes* and *Lachlania* show that the lowest mean oxygen saturation where both *Leptohyphes* and *Lachlania* occur corresponds to an average altitude of 3064 m a.s.l. (Figure 2) – according to the linear regression between mean oxygen saturation vs. altitude extracted from Jacobsen (2008a). In terms of mean water temperature, *Leptohyphes* lower limit of 9.0 °C on average corresponds to 3376 m a.s.l., while *Lachlania* 11.1 °C corresponds to 3021 m a.s.l. (Figure 2), based on the relationship between the mean temperature and altitude also found in Jacobsen (2008a).

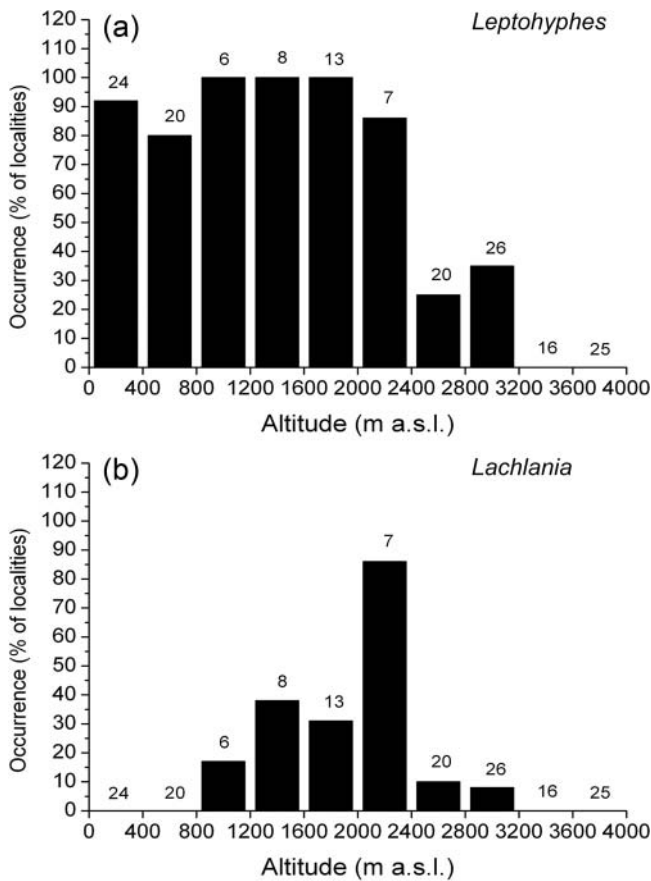


Figure 1. Percentage occurrence of *Leptohyphes* Eaton, 1882 (a) and *Lachlania* Hagen, 1868 (b) in streams in discrete altitude classes from 400 to 4000 m a.s.l. Numbers above columns denote the number of stream sites included in each category. Mainly data from Jacobsen (2004, 2008a).

Survey along Río Guapal

All site characteristics varied considerably between localities (Table 1), but only mean current and mean stream width were significantly (negatively) correlated with altitude ($p < 0.05$) (Table 2). Oxygen concentration and conductivity showed a tendency to decrease towards higher altitudes (Table 2). Substratum composition, benthic detritus, and algal biomass did not vary systematically along the stream reach.

Within this limited altitudinal range, neither the overall density nor the rarefied taxon richness were correlated with altitude (Figure 3, Table 2). In contrast, the benthic density of both *Leptohyphes* and *Lachlania* decreased significantly with increasing altitude (Table 2), and both taxa disappeared between site 3 (2950 m) and site 4 (3080 m) (Figure 3). *Leptohyphes* density was positively related to mean current and mean stream width while that of *Lachlania* was positively related to mean water depth. These variables were correlated with each other and with altitude (Table 2).

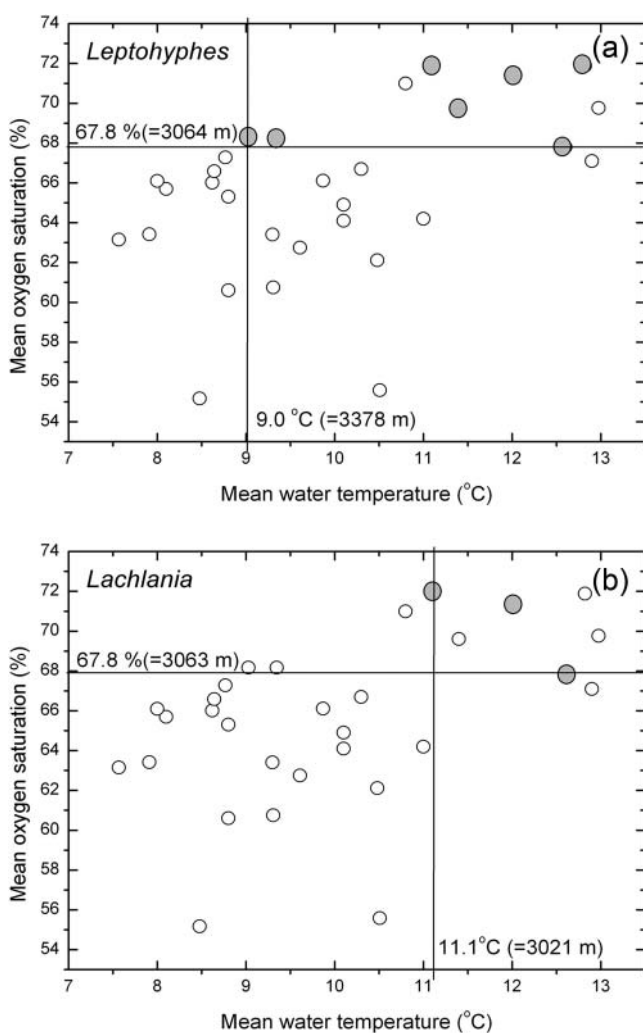


Figure 2. Mean water temperature and oxygen saturation in the 30 stream sites included in Jacobsen (2008a) and the occurrence of *Leptohyphes* Eaton, 1882 (a) and *Lachlania* Hagen, 1868 (b) marked by filled circles. The vertical line marks the lowest water temperature where these taxa were found and the altitude that these values correspond to from linear regressions of temperature vs. altitude obtained from Jacobsen (2008a). The horizontal line marks the lowest oxygen saturation where these taxa were found and the altitude that these values correspond to from linear regressions of oxygen vs. altitude obtained from Jacobsen (2008a).

Few adults were caught. At the downstream site, 1 individual of *Leptohyphes* was caught in the light trap and 2 were collected in the sticky traps, while 10 individuals of *Lachlania* were collected by hand netting. At the upstream site, no adults of these two taxa were caught. So, for both taxa, adults were found at the lowest site where the nymphs were present in the stream, but not at the upper site where their nymphs were absent.

Table 1. Environmental characteristics from the five study sites along the Río Guapal.

	Site 1	Site 2	Site 3	Site 4	Site 5
Altitude (m a.s.l.)	2780	2875	2950	3080	3150
Temp (°C)	13.0	12.0	10.3	12.0	12.5
Oxygen (mg L ⁻¹)	7.68	7.69	8.05	7.62	7.8
Conductivity at 25°C (μS cm ⁻¹)	382	324	218	313	181
pH (units)	7.51	7.53	7.81	6.65	7.94
Mean current (m s ⁻¹)	0.47	0.39	0.14	0.07	0.09
Mean depth (m)	0.23	0.21	0.23	0.09	0.14
Mean width (m)	6.25	7.03	2.71	2.59	1.41
Boulders and cobbles (%)	59	50	40	73	67
Pebble and gravel (%)	29	39	33	7	27
Sand (%)	12	6	20	13	0
FPOM (g m ⁻²)	0.24	0.78	0.28	0.23	0.30
CPOM (g m ⁻²)	0.58	2.73	0.98	1.37	1.69
Suspended organic particles (mg L ⁻¹)	4.4	0.8	1.2	8.3	1.6
Chlorophyll a (mg m ⁻²)	22.6	19.6	8.3	7.2	23.2

Transfer experiment

Data logger temperature and oxygen data revealed highly significant differences between the two sites (Appendix S1) with approximately 2 °C of temperature difference ($t = 30.775$, $p < 0.001$) and ca. 5% difference in O₂ saturation ($t = 106.9975$, $p < 0.001$). Differences in oxygen concentration were smaller, ca. 0.16 mg/l, but highly significant ($t = 13.4649$, $p < 0.001$). The transfer experiment revealed a significantly lower survival of transferred nymphs compared to controls (two-way ANOVA on slopes, $p = 0.005$, Table 3, Figure 4). We found no difference of survival response through time between the two taxa (two-way ANOVA on slopes, $p = 0.683$, Table 3).

Further *post hoc* tests of survival data for a given exposure time revealed that exposed individuals of *Lachlania* differed from control ones only after nine days of exposure whereas these differences occurred after the third day of exposure for *Leptohyphes*.

Table 2. Correlation coefficients (Pearson product-moment) between environmental and fauna parameters along the five study sites in Río Guapal. Bold denotes significant correlations ($p < 0.05$).

	Altitude (m a.s.l.)	Log density (ind. m ⁻²)	Number of taxa	Rarefied taxa number	Log <i>Leptohyphes</i> (ind. m ⁻²)	Log <i>Lachlania</i> (ind. m ⁻²)
Altitude (m a.s.l.)	1.00	-0.06	-0.78	-0.33	-0.99	-0.92
Temperature (°C)	-0.11	0.55	-0.26	-0.68	0.23	-0.22
Oxygen (mg L ⁻¹)	-0.76	-0.20	0.93	0.81	0.69	0.84
Conductivity (μS cm ⁻¹)	-0.74	-0.09	0.68	0.27	0.81	0.44
pH (units)	-0.09	0.58	-0.30	-0.43	0.02	0.35
Mean current (m s ⁻¹)	-0.92	0.39	0.61	-0.03	0.96	0.77
Mean depth (m)	-0.82	0.23	0.50	0.17	0.74	0.95
Mean width (m)	-0.88	0.31	0.77	0.12	0.94	0.74
Boulders and cobbles (%)	0.53	0.04	-0.54	-0.45	-0.43	-0.82
Pebble and gravel (%)	-0.53	0.58	0.30	-0.14	0.50	0.73
Sand (%)	-0.34	-0.88	0.53	0.93	0.22	0.42
FPOM (g m ⁻²)	-0.28	0.54	0.48	-0.09	0.36	0.34
CPOM (g m ⁻²)	0.18	0.53	0.13	-0.27	-0.08	-0.12
Susp. org. part. (mg L ⁻¹)	0.17	-0.60	0.00	0.28	-0.13	-0.46
Chlorophyll a (mg m ⁻²)	-0.23	0.92	-0.28	-0.84	0.31	0.14

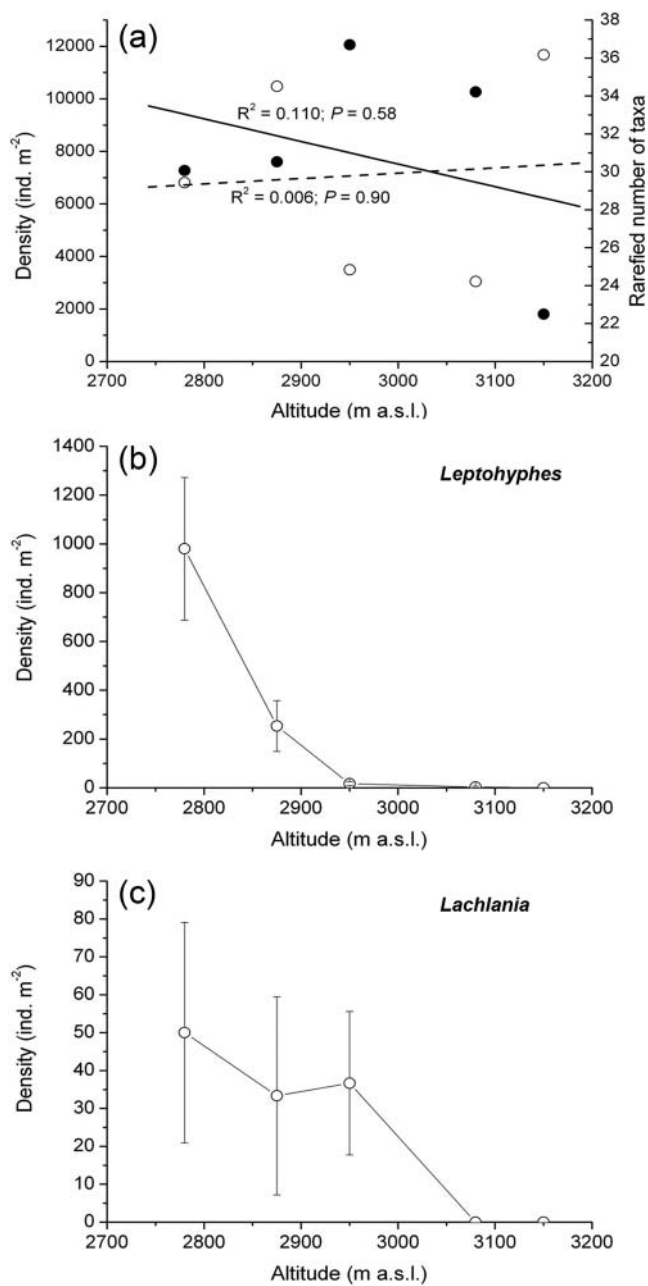


Figure 3. Density (open circles and dashed regression line) and rarefied number of taxa (filled circles and continuous line) of the total benthic macroinvertebrate fauna in relation to altitude in the Río Guapal (a). Density of *Leptohyphes* Eaton, 1882 (b) and *Lachlania* Hagen, 1868 (c) nymphs in relation to altitude. Error bars denote S.E.

Table 3. Results of the two-way ANOVA analysis on slopes of survival vs. exposure time (see Methods, $N = 4$ replicates for each species and each treatment) for *Leptohyphes* Eaton, 1882 and *Lachlania* Hagen, 1868 in both the exposed (high altitude) and the control site. * denotes significant effect of factor on slopes.

Factor	Df	Sum sq. $\times 10^4$	Mean sq. $\times 10^4$	F value	P value
Treatment	1	79.477	79.477	11.8859	0.005 *
Taxa	1	1.166	1.166	0.1744	0.683
Treatment \times taxa	1	3.667	3.667	0.5484	0.473
Residuals	12	80.240	6.687		

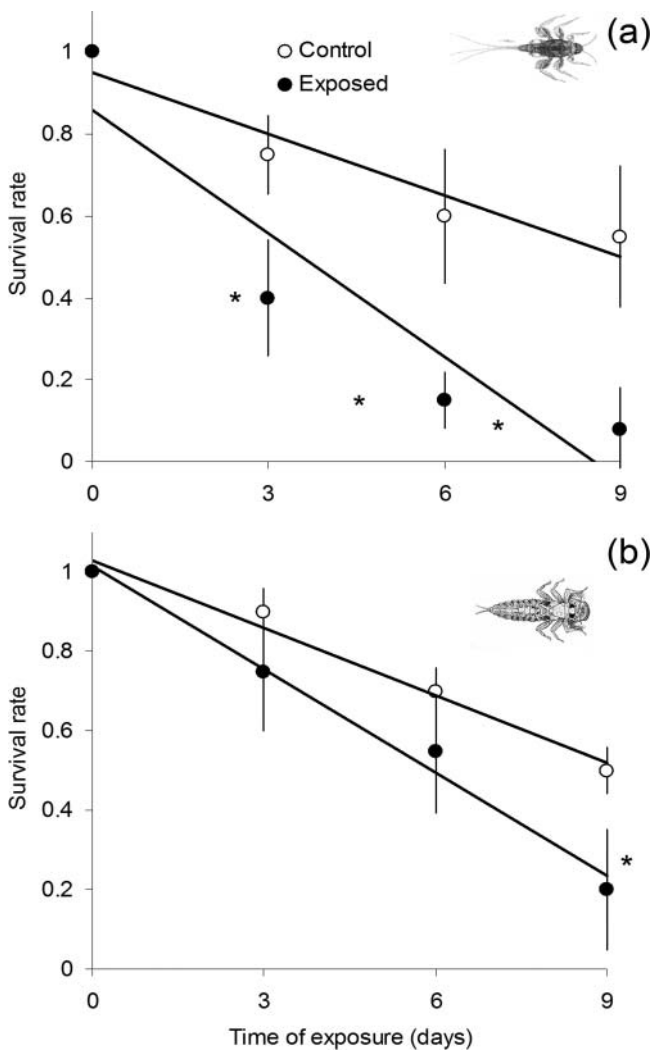


Figure 4. Survival rates for *Leptohyphes* Eaton, 1882 (a) and *Lachlania* Hagen, 1868 (b) and in both the exposed (high altitude) and the control site after three, six, and nine days of exposure. Linear models adjusted to the mean survival values (\pm SD, $N = 4$) are shown. * denote significant differences between treatments at the same exposure time using *post hoc* Tukey test ($p < 0.05$).

(Figure 4). For both taxa, the survival rate was significantly higher for large individuals (ANCOVA, *Leptohyphes* MSS = 22.59, $F = 32.45$, $p < 0.001$, *Lachlania* MSS = 19.14, $F = 19.21$, $p < 0.001$).

Discussion

In the Río Guapal, overall macroinvertebrate parameters, such as density and richness, did not change over the narrow altitudinal range studied. Nevertheless, densities of *Leptohyphes* and *Lachlania* declined gradually along the altitudinal range and these taxa disappeared completely between 2950 and 3080 m a.s.l. This corresponds perfectly with the extensive data-set on distribution of *Leptohyphes* and *Lachlania* shown in Figure 1, and therefore, indicates that our findings have general validity and that they can be expanded to a larger geographic scale, in spite of the lack of replicated streams in this study.

Densities of *Leptohyphes* and *Lachlania* were significantly correlated to stream-size-related variables, such as current, width (*Leptohyphes*), and depth (*Lachlania*). These variables might, of course, be causal for the distribution of these taxa, but they themselves are correlated with altitude. We do not have sufficient data from streams at lower altitudes to test for possible relations to current, width, and depth, independent of altitude. Otherwise, no other significant relationships were found between benthic densities and site characteristics, which was not surprising considering the low number and the characteristics of study sites. It is even less likely to demonstrate relationships with elusive variables, such as dissolved oxygen and water temperature represented by spot measurements. Nevertheless, it is important to note that the two-week temperature and oxygen saturation data revealed significant differences between the two extreme sites. This – plus the fact that the upper altitudinal limit of the two taxa in the Río Guapal coincides with the altitude that generally corresponds to the lowest mean oxygen saturation where the two genera are found (Figure 2) – suggests that oxygen saturation (= partial pressure) and perhaps temperature (particularly in the case of *Lachlania*) are related to the altitudinal limit of these taxa. Jacobsen, Rostgaard, and Vásquez (2003) and Jacobsen (2008a) suggested that low partial pressure of oxygen in water might be an important factor excluding certain species from high altitude streams. Also, in the Río Guapal, oxygen might be interacting with current in determining this limit. The upper site showed a marked reduction in mean current velocity and previous studies have revealed a strong, although complex, relationship between this variable and oxygen renewal rate (Statzner 2008). This might be especially important for active filter feeders, like *Lachlania*, which are strongly dependent on high current velocity (Baptista et al. 2006). However, this is probably the case only in streams, like the Río Guapal, which are characterised by small, slow flowing headwaters. Tropical high altitude streams are very variable in channel form and appearance (Jacobsen 2008b), so implying that slow velocity is a general cause of species disappearance in tropical headwaters might not be correct.

Jacobsen and Brodersen (2008) measured respiration of larvae of six insect genera (including *Lachlania* sp.) from Ecuadorian Andean streams in relation to oxygen saturation and temperature to determine whether natural altitudinal limits of the six genera were reflected as physiological critical points on respiration curves. Altitudinal limits of the six genera, defined by the lowest mean temperature and oxygen saturation at which

they were encountered in the field, were not reflected as critical points on their respiration curves. Therefore, neither the temperature nor oxygen conditions at the genera's altitudinal limits could be attributed to absolute short-term physiological tolerances. However, at high altitude, insects experience constant lower temperature and oxygen than at low altitude, and this has been shown to cause reduced metabolism, longer hatching times, and greater mortality in several terrestrial insects (Dillon, Frazier, and Dudley 2006, and references therein). Therefore, reduced temperature and/or oxygen are likely to, at least partly, compromise fitness in the long term by reducing feeding, growth, emergence, and fecundity of aquatic insects. In our transplantation experiment of aquatic nymphs, we found considerable mortality in both control and transplanted nymphs, probably due to handling stress and sub-optimal conditions inside cages. Nevertheless, there was a significantly lower short-term survival of transferred *Leptohyphes* and *Lachlania* compared to controls. This suggests that aquatic insects are limited by altitude-related factors at the juvenile stage and indicates that at least some of the altitudinal constraint is related to the aquatic abiotic environment rather than to biotic interactions with predators or competitors. Larval mortality in our experiment might have been enhanced by a reduction in current inside the fine mesh cages, which was an inevitable consequence of our experiment. However, note that *Lachlania*, an active filterer according to Baptista et al. (2006), and thus, a taxon highly dependent on fast flow, responded more slowly to the experiment than *Leptohyphes*. Also, as stated in the Methods section, we made an effort to find similar places, with swift current in both streams. Therefore, although mean current in the upper site was low, there were some microhabitats with faster current. This probably helped reduce the effect of current on insect survival. The significant effect of the nymph size on mortality (large nymphs having highest survival rates) is interesting because, although larger individuals are probably better at tolerating physical handling, they should also have higher rates of oxygen consumption (Hemmingsen 1960; Hamburger and Dall 1990; Rostgaard and Jacobsen 2005) and be more affected by the reduced current.

Very few adults of *Leptohyphes* and *Lachlania* were collected in this study. We propose two possible explanations for this. First, light traps at high altitudes might not be optimal because air temperatures at sunset and sunrise are probably too low for flight activities of mayflies. Second, sampling effort with hand nets might not have been high enough for sparse adult populations with asynchronous population dynamics. Even though there is very little information regarding flight patterns of adults of tropical aquatic insects (Rios-Touma, Encalada, and Prat 2012), several authors have suggested that a multivoltinism strategy, with asynchronous periods of adult emergence, would be the most beneficial strategy in environments, like tropical, high-altitude habitats, where diel variations in temperature are more pronounced than seasonal variations (Guhl 1989; Luteyn and Churchill 1999; Jacobsen 2008b; Rios-Touma et al. 2012). In fact, in their survey of adults during the wet and dry seasons in a tropical high-Andean stream, Rios-Touma and her collaborators (2012) found no differences in the number of adults flying between the two seasons. Also, mating in mayflies is based on male swarming which usually takes place near conspicuous features (landmarks) and at certain periods of the day (Allan and Flecker 1989), that might not have been sampled during our hand-net sampling. It is important to note that sampling of adults in our study was carried out during the dry season, but given the previously mentioned evidence, we do not think that this could have been the reason for the low number of adults we collected. Adults of our studied taxa

were non-abundant in comparison to adults of Diptera collected during the same period and with the same methods (V. Crespo-Pérez, personal observation), and this coincides with that found in previous studies in Andean streams (Wolf, Matthias, and Roldan 1988; Flint 1991; Rios-Touma et al. 2012).

The adults were collected where the nymphs were found in the stream, but not at the upstream site where the aquatic nymphs were absent. Similar data from two other streams in the area, each with a downstream site (2840 and 2850 m a.s.l.) with nymphs and adults and an upstream site (3070 and 3360 m a.s.l.) with neither nymphs nor adults (Crespo-Pérez 2007) indicate that the absence of adults at the upstream site was not just due to insufficient collecting effort. This is in accordance with Winterbourn and Crowe (2001) who found a close correlation between larval and adult populations of insects along a New Zealand mountain stream. In lowland streams, most individuals of adult stream insects disperse less than about 50 m laterally from the stream (Sode and Wiberg-Larsen 1993; Petersen, Masters, Hildrew, and Ormerod 2004), and even though upstream migration predominates for most groups (Madsen, Bengtson, and Butz 1977; Winterbourn and Crowe 2001; Winterbourn, Chadderton, Entrekin, Tank, and Harding 2007), distances travelled upstream rarely exceed 700 m (Macneale, Peckarsky, and Likens 2005). Little is known about the insect flight behaviour in highlands (there is practically no published information about this subject from tropical highlands), and the few existing studies are contradictory, with one revealing long-distance upstream dispersal of adults up to the snowline (Thomas 1975), and others revealing considerably reduced flight in highland compared to lowland areas (Mani 1968; Hodkinson 2005). In the specific case of mayflies, they are often described as 'poor fliers' (Edmunds Jr, Jensen, and Berner 1976; Alexander 2007), although there is some evidence of longer range dispersal (Hughes, Bunn, Cleary, and Hurwood 2000) and of rapid flight and precise manoeuvring during aerial mating (Sartori, Keller, Thomas, and Passera 1992). Nevertheless, the short life spans of adults (from less than 90 minutes to a few days, Merrit and Cummins 1996), the morphology of their wings, developed for passive windborne transport, and their use of active flight for male swarming and female oviposition (Brittain 1978; Sartori et al. 1992) suggest that they are not especially built for active long-distance flight (Malmqvist 2000). Consequently, even though long-distance dispersal of up to several kilometres of few individuals does occur (Hughes et al. 2000) and studies of population genetics show that adult flight is the major mechanism of dispersal (Hughes, Schmidt, McLean, and Wheatley 2008), our finding of no adults at sites with no nymphs was not surprising. It is also important to consider that females of many aquatic taxa have been found to oviposit selectively in places that minimize egg and larval mortality (Gillett 1971; Baba and Takaoka 1991; Peckarsky, Taylor, and Caudill 2000; Lancaster, Downes, and Reich 2003; Reich and Downes 2003a, 2003b; Encalada and Peckarsky 2006; Binckley and Resetarits 2008). Given the hydromorphological differences between the two extreme sites of Rio Guapal, it is possible to hypothesize that female adults do not fly to the highest site because a narrow, shallow, slow flowing stream might not be ideal for her offspring. However, data about specialized oviposition in high altitude, tropical streams is very scarce and more studies should be carried out on this subject to obtain conclusive answers (Rios-Touma et al. 2012). A final possibility is that very sparse populations of just a few individuals of the species in the upper part of the studied reach may be a problem when males and females need to mate, thus, limiting recruitment and population persistence. In any case, the fact

that we did not find any adults of either *Leptohyphes* or *Lachlania* in the upper site suggests that at least some of the altitudinal constraint must lie at the adult stage as well.

In conclusion, the combined approaches in this study have provided further insight into the factors limiting aquatic insect distribution along altitudinal gradients. In addition, our results support the hypothesis that altitudinal limits are related to the abiotic environment, one of the four mechanisms of community organisation on gradients initially proposed by Whittaker (1967). Apparently, constraints for aquatic insect distribution lie at both the juvenile and adult stages. Even though this and previous studies have already focused on the possible limiting factors along altitudinal gradients in tropical mountains, with a special focus on oxygen availability along the gradient (Jacobsen 2003, 2004), we still do not know whether exclusion from higher altitudes is driven by oxygen, temperature, a combination of these, or some other factors, more stream specific, like current velocity. Oxygen saturation and temperature are usually highly correlated along altitudinal gradients (Jacobsen 2008a) and their specific effects upon diversity and community patterns are thus, difficult to disentangle. Also, current has been found to influence oxygen supply by increasing the renewal rate in water, but the relationship between these and invertebrate respiration is complex (Statzner 2008). Finally, we need to understand the role of simple preference for certain environmental conditions, fecundity, and biotic interactions for the long-term survival and gradual decline in population densities approaching altitudinal limits.

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