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Pesticide contamination drives adaptive genetic variation in the endemic mayfly *Andesiops torrens* within a semi-arid agricultural watershed of Chile☆

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ABSTRACT

Agrichemical contamination can provoke evolutionary responses in freshwater populations. It is a particularly relevant issue in semi-arid regions due to the sensitivity of endemic species to pollutants and to interactions with temperature stress. This paper investigates the presence of pesticides in rivers within a semi-arid agricultural watershed of Chile, testing for their effects on population genetic characteristics of the endemic mayfly *Andesiops torrens* (Insecta, Ephemeroptera). Pesticides were detected in sediment samples in ten out of the 30 sites analyzed throughout the upper part of the Limarí watershed. To study the evolutionary impact of such contamination on *A. torrens*, we used a genome-wide approach and analyzed 2056 single nucleotide polymorphisms (SNPs) loci in 551 individuals from all sites. Genetic differentiation was weak between populations, suggesting high gene flow across the study area. While we did not find evidence of pesticide effects on genetic diversity nor on population differentiation, the allele frequency of three outlier SNP loci correlated significantly with pesticide occurrence. Interrogation of genomic resources indicates that two of these SNPs are located within functional genes that encode for the low-density lipoprotein receptor-related protein 2 and Dumpy, both potentially involved in insect cuticle resistance processes. Such genomic signatures of local adaptation are indicative of past adverse effects of pesticide exposure on the locally adapted populations. Our results reveal that *A. torrens* is sensitive to pesticide exposure, but that a high gene flow may confer resilience to contamination. This research supports the contention that *A. torrens* is an ideal model organism to study evolutionary responses induced by pesticides on non-target, endemic species.

1. Introduction

Contamination of freshwater ecosystems by chemical pollutants derived from industrial or agricultural activities has emerged as a major environmental problem (Kohler and Triebskorn, 2013), whose harmful effects have been documented across all levels of biological complexity (Beketov et al., 2013; Kuzmanovic et al., 2017; Russo et al., 2018). This may be particularly true for biota that live in freshwater ecosystems (Geist, 2011), especially those experiencing agricultural, and more specifically pesticide, contamination (Kohler and Triebskorn, 2013; Schäfer et al., 2011).

Agrichemicals are very common pollutants in freshwaters (Aktar et al., 2009), and often enter natural waterbodies through precipitation events followed by surface runoff (Knight et al., 2013; Liess et al., 1999). They can cause a wide range of direct and indirect effects on aquatic organisms (Schäfer et al., 2011), that can be

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triggered at low concentrations and delayed substantially relative to exposure (Russo et al., 2018). Recently, there has been an increasing awareness that the genetic impacts of pollutants can be long-lasting and lead to multigenerational changes, being thus able to drive contemporary evolution (Bickham et al., 2000; Bickham, 2011; Brady et al., 2017). Genetic responses at the population level include increased mutation rates and changes in genome-wide genetic diversity and allelic or genotypic frequencies due to contaminant-mediated selective pressures and altered gene flow between populations (Bickham, 2011; Brady et al., 2017). Even though our knowledge of the genetic effects of pesticides on freshwater species populations is still in its infancy, empirical evidence indicates that these effects are capable of altering neutral genetic diversity and structure (Inostroza et al., 2016, 2018) and can drive adaptive genetic variation (Major et al., 2018; Shahid et al., 2018; Weston et al., 2013). This suggests that agrichemical contamination may be a key driver of evolutionary change in freshwater environments.

The evolutionary impact of agrichemical contamination is an issue of special concern for species in semi-arid climates. As it is recognized that temperature stress can increase the sensitivity of aquatic organisms to such exposure (Liess et al., 2016), temperature-pesticide interactions are thus likely to occur in agricultural watersheds from regions of Mediterranean and semi-arid climates where water temperature increases substantially during summer. In Chile, agriculture essentially aggregates in such climates developing along river basins within the central region of the country (Meza et al., 2012). Accordingly, pesticide occurrence has been mainly reported in river basins and lakes of central and south-central Chile (Baéz et al., 1996; Barra et al., 2001a, 2001b; Climent et al., 2018; Cooman et al., 2005; Montory et al., 2017; Retamal et al., 2013); with an additional case in the southern Traiguen river (Palma et al., 2004). However, information relative to pesticide contamination is scarce in more arid parts of Chile, located northward. In semi-arid agricultural watersheds of the Coquimbo region (north-central Chile), recent evidence demonstrates that pesticides are released in the environment and impact human health (Corral et al., 2017), indicating that pesticide contamination is an environmental issue in this region. Yet, the only evidence of freshwater contamination comes from a 2004 report from the Chilean Water Management Agency (Dirección General de Aguas, DGA) revealing the presence of pesticides downstream of the Limarí watershed (information summarized in Diez, 2010). In addition, information on the impact of such contamination on the local aquatic fauna is lacking in Chile.

Given the context described above, the aims of this study were to i) test for the presence of pesticides in river bodies upstream of the semi-arid Limarí watershed located in the Coquimbo region in north-central Chile, and ii) evaluate their potential effect, combined with other environmental and land-use variables, on genetic differentiation of populations of the endemic mayfly *Andesiops torrens* (Insecta, Ephemeroptera). Mayfly species are recognized bio-indicators of anthropogenic disturbances (Firmiano et al., 2017) and represent interesting targets for environmental monitoring. The species *A. torrens* has a wide distribution range in Chile, between 30°S and 54°S, is abundant and plays a functional role as filter feeder/collector in well-oxygenated high-slope rivers (Sabando et al., 2011). To investigate the genetic effects of pesticides on the mayfly *A. torrens*, we used a SNP dataset generated by next-generation sequencing (NGS). NGS is opening novel investigations of genetic responses to contaminant exposure in non-model organisms (Oziolor et al., 2016, 2017). By providing rapid and cost-effective generation of millions of reads from thousands of loci generated by restriction site-associated DNA sequencing (RADseq; Andrews et al., 2016), it is possible to not only investigate neutral demographic processes such as gene flow and genetic drift, but also to identify potential signatures of selection within a species genome via the evaluation of putatively adaptive genetic variation (Catchen et al., 2017; Manel and Holderegger, 2013). RADseq markers have been successfully applied for the detection of genetic loci influenced by water pollutants, showing their great potential for understanding the forces driving aquatic organism evolution in response to such threats (Rusconi et al., 2018).

2. Materials and methods

2.1. Study area and Andesiops torrens sampling

The Limarí watershed is located within the Coquimbo region in North-Central Chile (Fig. 1), and is characterized by a semi-arid climate (Falvey and Garreaud, 2007). Most water resources of the region originate in the Andes cordillera, either from direct runoff during rainstorms or from snow melt. The seasonal cycle of precipitation is pronounced, with precipitation events occurring almost exclusively during the winter months of June to August. The contribution of precipitations to surface runoff is of prime importance in this region, as the glacier contribution is actually quite negligible (Nicholson et al., 2009).

Agriculture has increased radically within the Limarí watershed over the last two decades, becoming the main economic activity (Squeo et al., 2001). Currently over 28.500 ha within the watershed are agricultural, representing more than 50% of the total agricultural area within the Coquimbo region (http://promus.prommra.cl/sup-historica/limari/montepatria/sup-historica-limari-2000.php). The upper part of the Limarí watershed is mainly dedicated to the production of fruit trees (citruses, avocado), walnuts and grapes, with a cultivated area of 7.279 ha above the village of Monte Patria. The study area is located in the pre-cordillera, between 450 and 1500 m above sea level. Cultivated areas within the study area are characterized by fringes of intensive agriculture distributed alongside the river, surrounded by steep rocky slopes (see Fig. 1 and Appendix 1). Most pesticides in this area are applied between September and February.

For this study, 30 sites were selected along the upper part of the Limarí watershed (Fig. 1, Table 1). Sampling of *A. torrens* mayflies was carried out between May and June 2015, that is at the end of a 5-year megadrought period (Garreaud et al., 2017). Fifteen to twenty individuals were sampled by hand per study site, using featherweight entomological forceps to remove each of the small specimens (~5 mm length) from rocks gathered from the river bed. After collection, the mayflies were stored in 95% alcohol at 4 °C until DNA extraction.

2.2. Molecular analyses and bioinformatics

2.2.1. DNA extraction and quantification

Genomic DNA was extracted using a CTAB protocol described in Chen et al. (2010), except the RNase treatment that was composed of a mix of 400 μg RNase A (Sigma-Aldrich) and 200 U of RNase T1 (Thermo Fisher Scientific). DNA was resuspended in water, checked for integrity on 1% agarose gels, quantified by spectrofluorometry with the Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher Scientific) using a microplate reader Victor X2 (PerkinElmer), and stored at −20 °C.

2.2.2. Genotyping-by-sequencing using NextRAD

A total of 570 DNA samples were shipped to the SNPsaurus (LLC) laboratory (Eugene, Oregon, USA), where genomic DNA was converted into nextRAD genotyping-by-sequencing libraries as in

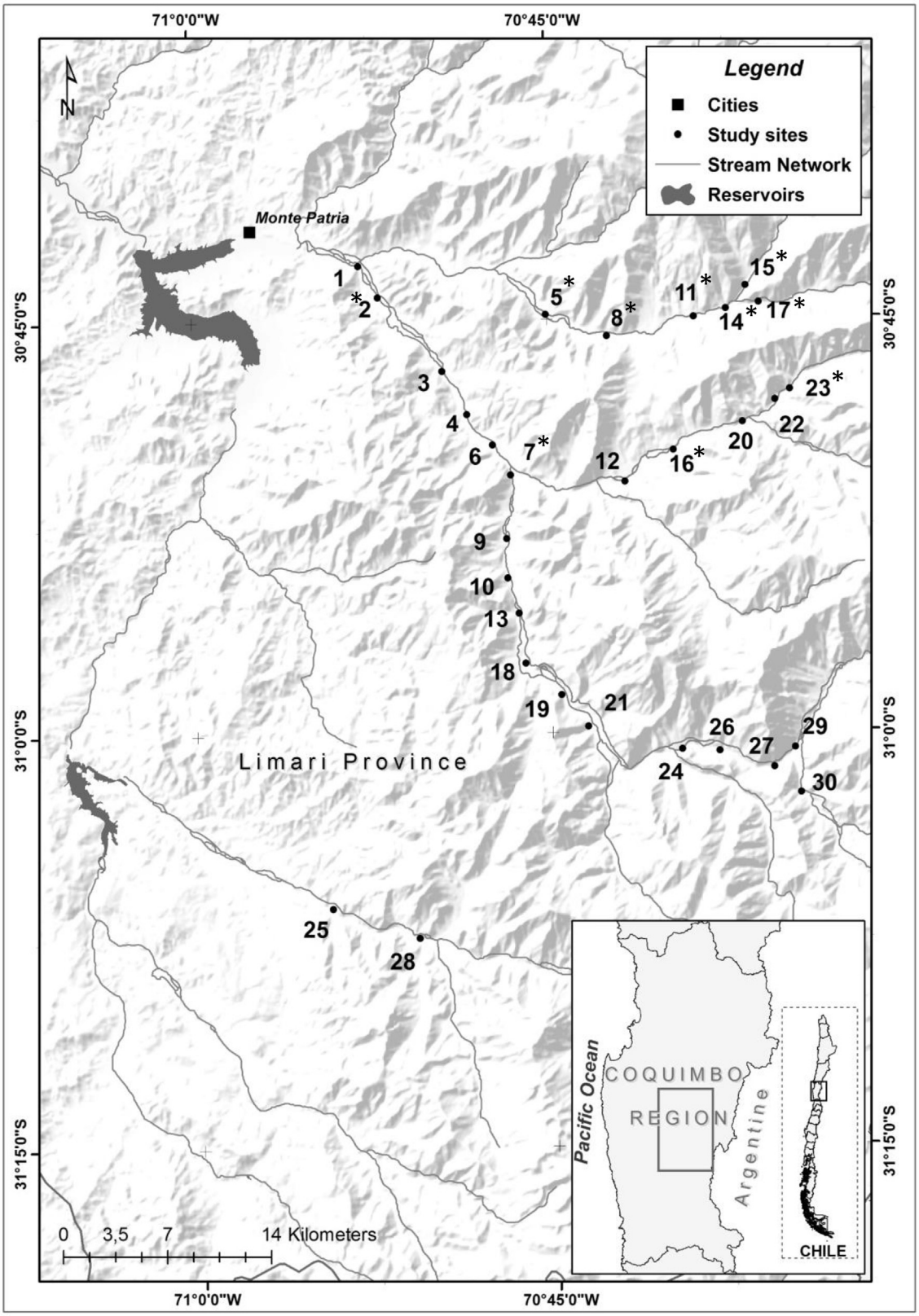

Fig. 1. Topographic map showing the 30 study sites within the Limarí river basin in Chile. Sites are coded by number as described in Table 1. Sites where pesticides were detected above method detection limits are indicated with an asterisk.

Table 1
Information about the study sites and pesticide detection in sediment samples along the Limarí basin during the October 2016 campaign. #: site code. Pesticide quantification values above method detection limits are reported in bold and expressed in ng/g of dry weight of sediment (detection limits are described in Appendix 4).

#	Site name	Catchment	Latitude	Longitude	Sediments (ng/g)			
					Atrazine	DEA	Carbofuran	Permethrin
1	Las Juntas	Río Grande	−30.717	−70.882	0.00	0.00	0.00	0.00
2	Garreton	Río Grande	−30.736	−70.869	**0.32**	0.00	0.00	0.00
3	Panguecillo	Río Grande	−30.781	−70.824	0.00	0.00	0.00	0.00
4	Peñon	Río Grande	−30.808	−70.807	0.00	0.00	0.00	0.00
5	Barrancones	Río Rapel	−30.748	−70.751	**0.40**	**2.28**	0.00	0.00
6	Semita	Río Grande	−30.826	−70.789	0.00	0.00	0.00	0.00
7	Gallardina	Río Grande	−30.844	−70.777	0.00	0.00	0.00	**1.01**
8	Sol Praderas	Río Rapel	−30.761	−70.708	**0.79**	**7.49**	**1.11**	0.00
9	Pulpica	Río Grande	−30.883	−70.780	0.00	0.00	0.00	0.00
10	Vado Hondo	Río Grande	−30.907	−70.780	0.00	0.00	0.00	0.00
11	Mollacas	Río Rapel	−30.750	−70.647	**0.40**	**1.70**	0.00	0.00
12	Pedregal	Río Mostazal	−30.849	−70.697	0.00	0.00	0.00	0.00
13	Cuyano	Río Grande	−30.928	−70.772	0.00	0.00	0.00	0.00
14	Molles 1	Río Rapel	−30.745	−70.624	**1.35**	0.00	**0.72**	0.00
15	Palomo	Río Rapel	−30.731	−70.610	**0.48**	**3.03**	0.00	0.00
16	Maqui	Río Mostazal	−30.830	−70.662	**0.42**	0.00	0.00	0.00
17	Molles 2	Río Rapel	−30.742	−70.601	**1.47**	0.00	0.00	0.00
18	Cisterna	Río Grande	−30.958	−70.768	0.00	0.00	0.00	0.00
19	Tulahuen 1	Río Grande	−30.978	−70.743	0.00	0.00	0.00	0.00
20	Maiten 1	Río Mostazal	−30.814	−70.613	0.00	0.00	0.00	0.00
21	Tulahuen 2	Río Grande	−30.997	−70.725	0.00	0.00	0.00	0.00
22	Maiten 2	Río Mostazal	−30.800	−70.590	0.00	0.00	0.00	0.00
23	Maiten 3	Río Mostazal	−30.794	−70.580	**1.15**	0.00	**4.01**	0.00
24	Tulahuen 3	Río Grande	−31.011	−70.659	0.00	0.00	0.00	0.00
25	Barrancas	Río Cogotí	−31.106	−70.907	0.00	0.00	0.00	0.00
26	Tulahuen 4	Río Grande	−31.013	−70.633	0.00	0.00	0.00	0.00
27	Ramadas 1	Río Grande	−31.023	−70.594	0.00	0.00	0.00	0.00
28	Durazno	Río Cogotí	−31.124	−70.846	0.00	0.00	0.00	0.00
29	Ramadas 2	Río Grande	−31.011	−70.579	0.00	0.00	0.00	0.00
30	Turbio	Río Turbio	−31.038	−70.575	0.00	0.00	0.00	0.00

Russello et al. (2015). The nextRAD libraries were multiplexed with 190 samples randomly assigned to each of three Illumina lanes and sequenced on a HiSeq 4000 platform as 75 bp single-end reads (University of Oregon). Detailed procedures for library production and data pre-filtering performed by the SNPsaurus staff are described in Appendix 2.

We then used VCFtools (Danecek et al., 2011) to further filter the SNP dataset. We first removed individuals with more than 40% missing data, a cut-off level with no apparent effect on population genetic diversity and demographic estimates (Pfeiffer et al., 2018; Shafer et al., 2017). Then, we kept loci that were genotyped successfully in at least 70% of the individuals, with a base quality $\geq$30 and a minor allele count of 3 within a range of 10−100 mean depth. Because some individuals were removed from the initial file, we filtered again all the loci for a minimum allele frequency of 0.05 across all individuals. We then excluded sites with indels, and also removed those with observed heterozygosity >0.5 to avoid potential paralogues (Hohenlohe et al., 2011). All loci were tested for departure from Hardy-Weinberg equilibrium using the exact test implemented in dDocent (Puritz et al., 2014). Loci showing significant departure from Hardy-Weinberg equilibrium ($P \leq 0.05$) in more than 30% of the sampling locations were excluded. Linkage disequilibrium was assessed using PLINK v1.7 (Purcell et al., 2007) and loci with $r > 0.7$ were discarded. Finally, we removed loci with more than 30% missing data by sampling site using dDocent. The filtering steps and resulting SNP counts are summarized in Table 2.

2.3. Environmental and spatial variables

2.3.1. General habitat description and spatial variables

At each site, we measured longitude, latitude and altitude using a portable GPS device. In addition, we calculated the distance to the La Paloma dam near Monte Patria (Fig. 1) as a proxy of the position of each site along the watershed. Several habitat characteristics were also described at each site during the sampling of *A. torrens* specimens. These include the river bank and channel widths, the proportion of aquatic vegetation, bryophytes and algae, as well as river substrate features such as the relative proportion of mud, gravel, cobble, boulder, and bedrock. Basic water physicochemical parameters were measured at three different time points during 2-day field campaigns performed during July 2015, October 2015 and January 2016. We considered the mean value for all estimated parameters, and the maximum and/or minimum registered value depending on the parameter (see detailed list of the environmental variables in Appendix 3). Dissolved oxygen, electrical conductivity, turbidity and water temperature were measured on site using a portable logging multiparameter meter HI9829 (Hanna Instruments). Water velocity was evaluated using a digital water velocity meter FP111 (Global Water) by taking independent measures at five different sectors within each sampling site, placing the flow probe at 10 cm above the river bottom. River depth was measured with the same equipment and protocol. For this specific parameter, we considered the average value across the three chemical sampling campaigns, plus the average and maximum values registered during the biological sampling during the May−June period.

A land use map of the study area was generated using previously digitized agricultural data from 2011 (CIREN, Santiago, Chile, https://www.ciren.cl/), actualized and completed by manual digitalization using GoogleEarth satellite pictures from 2015 (see Appendix 1). This map was used to calculate the surface devoted to agricultural activities above each site using a buffer area of 1000 m large x 2000 m long. We considered the area of all agricultural activities, including pasture, and the area dedicated to fruit production only, as these are subject to treatments with agrichemicals.

Table 2
Number of variant loci retained after each filtering step for *Andesiops torrens* within the Limarí basin. The number of filtered loci used for downstream statistical analyses is indicated in bold.

Step	SNP count
SNPsaurus (LLC) SNP catalogue	3817
Genotyped successfully in 70% of individuals. base quality ≥30. minor allele count of 3 within a range of 10–100 mean depth	3347
Minimum Allele Frequency of 0.05	3346
No indels	3098
No loci with $H_o > 0.5$	3039
HWE by population: exclude loci with $P \leq 0.05$ in more than 30% of populations	2780
Linkage disequilibrium between loci: $r \leq 0.7$	2682
Missing data by population <30%	**2056**
Outlier detection	
Bayescan	9
PCAdapt	82
Spatial AEM	111
Spatial MEM	100
Total unique outliers	**291**
Putatively neutral loci	**1765**

HWE: Hardy-Weinberg equilibrium. AEM: asymmetric eigenvector maps. MEM: Moran eigenvector maps.

2.3.2. Water and sediment chemical analyses

Water samples were collected three times during the 2-day field campaigns discussed previously. The samples were kept on ice in coolers and analyzed in a certified laboratory (Geoquímica, Coquimbo, Chile; ISO 9001:2008 certification) to quantify the concentrations of various nutrients and total levels of potentially toxic elements (aluminium, arsenic, calcium, copper, iron, magnesium and nitrates) using standard methods.

For the detection of pesticides, three sampling campaigns were carried out in October 2015, during a water pulse after heavy rainfall, January 2016 and October 2016. Each time, we collected river water samples in 1L amber glass bottles that were kept on ice in coolers and brought to the laboratory for solid phase extraction (SPE) within 32 h. SPE was performed using a vacuum manifold (UCT Inc.) and Clean-Up C18 1 g/6 mL cartridges (UCT Inc.), following procedures described in Appendix 2. Cartridges were wrapped in aluminium foil, stored in a plastic bag at 4 °C and shipped to the Water Sciences Laboratory at the University of Nebraska in Lincoln (USA) for posterior pesticide screening by gas chromatography-mass spectrometry (GC/MS). Because no residues of pesticides were detected in the October 2015 and January 2016 campaigns from water samples, sediments were also collected at each site jointly with the water samples during the October 2016 campaign. Sediment samples were placed in aluminium containers, stored in a cold cooler during the field campaign and at −20 °C in the laboratory. Five grams were then dried using a freeze dryer (VirTis adVantage Plus XL-70) and shipped in amber glass tubes to the Water Sciences Laboratory.

Pesticides were eluted from the SPE cartridges using 4 mL of ethyl acetate and followed processing similar to previously published methods (Cassada et al., 1994). Sediment-associated pesticides were extracted by microwave assisted solvent extraction (MASE) as described in Appendix 2. GC-MS analyses were carried out on an Agilent 5973 inert source instrument with a LEAP Technologies Combi-PAL autosampler. We screened for the presence of 26 pesticides based on compounds reported from previous investigations, as well as the likelihood of use within the region. These include: acetochlor, atrazine, bifenthrin, boscalid, carbofuran, chlorpyrifos, cyhalothrin lambda, cyprodinil, desethylatrazine (DEA), deisopropylatrazine (DIA), deltamethrin, diazinon, fludioxonil, malathion, methidathion, metolachlor, metribuzin, parathion ethyl, parathion methyl, pendimethalin, permethrin, propazine, pyrimethanil, quinoxyfen, tebuconazole, tefluthrin, triadimefon. Retention times, quantifier, confirming ions and method detection limits are reported for each compound in Appendix 4. Instrument detection limits, determined by repeated injection of the lowest standard, generally ranged between 10 and 371 pg injected, averaging 82 pg. Method detection limits were determined by extraction and analysis of 8–10 low-level fortified blanks according to EPA protocols for a low-level spike (USEPA, 2007). A more detailed protocol for the GC-MS analyses is available in Appendix 2.

Because pesticides were not detected in all the study sites, we considered three pesticide variables: pesticide presence, maximum pesticide concentration detected and cumulated pesticide concentration detected (considering the sum of all detected pesticides in a given site). All measures below detection limit were categorized as 0.

2.3.3. Environmental data reduction

A total of 43 variables were considered in this study (see detailed list and data in Appendix 3). Data from two metals were removed due to the lack of variation between sites (arsenic was not detected at any site while copper was elevated at only four sites and only one of the three samplings; Site 1: 0.10 mg/L, Site 3: 0.17 mg/L, and Site 15: 0.23 mg/L from the July 2015 sampling, and Site 27: 0.67 mg/L from the January 2016 sampling). To reduce the number of variables, we used a clustering of variables around latent variables (CLV) approach (Vigneau and Qannari, 2003) and selected the variable within each cluster that most strongly correlated with the group latent variable. The number of clusters was defined following Vigneau et al. (2015) recommendations.

2.4. Statistical analyses

2.4.1. Outlier detection

We identified loci not conforming to neutral expectations using population, individual and spatially-based approaches. We used the population-based Bayesian likelihood method implemented in the software BayeScan v.2.1 (Foll and Gaggiotti, 2008) to recognize F_{ST}-outliers. For each locus, BayeScan estimates the posterior probability of two competing models either including or not including selection. BayeScan was run with the default parameters and a total of 10,000 iterations. Outlier loci were identified using posterior odds and considering a false discovery rate (FDR) of 5%.

We also used pcadapt (Luu et al., 2017), an individual-based multivariate outlier detection method that identifies loci putatively under positive local selection. Because such loci tend to increase genetic differentiation, pcadapt considers loci that contribute significantly more to population structure than most loci

as candidate markers. We identified the optimal number of components (i.e., k-components) from the scree plot and used a 10% false discovery rate to identify outlier loci with significantly larger Mahalanobis distances.

Finally, we applied the Moran spectral outlier detection (MSOD) framework to look for spatial outlier loci not conforming to the spatial signature of gene flow (Wagner et al., 2017). This is done by identifying loci with unusual spatial power spectrum (i.e. the squared correlation coefficient between a locus and the spatial eigenvectors, Wagner et al., 2017). Before carrying out such analysis, it is necessary to generate spatial eigenvectors representing relevant dispersal dimensions of the organism under study. In *A. torrens* both downstream drift along the watercourses and active overland flying at the adult stage can occur. Therefore, we considered two alternative spatial structures, one representing non-directional overland processes and the other directional processes occurring from upstream to downstream along the watercourse. For the former spatial modeling, we applied the Moran eigenvector maps (MEM) framework (Dray et al., 2006). The spatial variables (the MEMs) were derived through the eigen-analysis of a symmetrical weighted connectivity matrix, which was constructed from a Euclidean distance-based connectivity scheme: the Delaunay triangulation (Dale and Fortin, 2010, Appendix 5). We weighted the non-zero links by the inverse geographic (Euclidean) distance between the connected site pairs so that the magnitude of the connectivity between linked sites was inversely proportional to their geographic distance. To model spatial processes occurring from upstream to downstream along the watercourse, we used the asymmetric eigenvector maps (AEM) framework (Blanchet et al., 2008). This method involves the eigen-analysis of a site-by-edges matrix coding for asymmetric, directional connections between sites, which we derived from the river network and by considering an upstream to downstream influence. As for the MEM construction, a weighting function was applied to the connected sites, corresponding in the present case to the inverse of the watercourse distance.

The MEM and AEM spatial vectors were used separately for the MSOD. For each locus, a MEM and AEM power spectrum were calculated and the deviation to the respective mean power spectrum of all loci was calculated as recommended by Wagner et al. (2017). Outlier loci were determined from the z-scores of the estimated deviations using a 5% cut-off level. To generate the set of neutral loci, we removed all the outliers detected by any of the performed methods (Table 2).

2.4.2. Population genetic diversity and differentiation analyses

Observed heterozygosity (Ho) was calculated with the R package *hierfstat*, which was also used to estimate allele richness using the function *allelic.richness*. Expected heterozygosity (He) and global population genetic differentiation estimates G_{ST} were assessed using the *genetic_diff* function of the vcfR v1.6.0 package available in R (www.r-project.org). Pairwise F_{ST} estimates between sampling sites were calculated with Arlequin V3.5.2.2 (Excoffier and Lischer, 2010), using 9999 permutations to assess significance. All the parameters mentioned above were estimated for both the complete and the neutral sets of loci, composed of 2056 and 1765 loci, respectively (Table 2). Since potentially adaptive loci can be useful for determining population structure (Batista et al., 2016), population genetic structure of *A. torrens* was evaluated on the complete set of loci by performing Discriminant Analysis of Principal Coordinates (DAPC; Jombart et al., 2010) with the Adegenet v2.0.0 package (Jombart and Ahmed, 2011) in R. Because no optimal *k* could be found using k-means clustering, DAPC was performed using sampled sites as predefined groups. The number of principal components (PCs) retained prior to discriminant analysis was defined using across-validation procedure (Jombart et al., 2010). We tested the groupings revealed by the DAPC analysis, and also the potential effects of the hydrographic structure and the presence of pesticides on the population genetic differentiation using analysis of molecular variance (AMOVA, Excoffier et al., 1992) in Arlequin V3.5.2.2. Mantel tests were carried out on both sets of loci using the *mantel* function of the Vegan v2.4-5 package in R to assess isolation by distance, using both Euclidian distances and hydrographic distances following the river network.

2.4.3. Detection of locus-environment associations

Loci potentially under selection were identified from the set of loci not conforming neutral expectations using an environmental association approach. First, we calculated Pearson correlations between the allele frequency of the outlier loci and the environmental predictors and used a false discovery rate of 10% to identify potential associations according to correlation t-tests. Because spatial autocorrelations in allele frequencies and environmental predictors can increase type I error rates when testing correlation significance (Legendre, 1993; Wagner et al., 2017), we quantified and tested the overall spatial autocorrelation of the loci and environmental variables for which we found a potential association as described in Biswas et al. (2017). For these analyses, we also used both the AEM and MEM spatial models (AEM or MEM). When significant spatial structure was detected, we re-tested the association between outliers and environmental predictors while controlling for spatial autocorrelation (Wagner et al., 2017) by performing a Moran spectral randomization test for correlation with 9999 randomizations (Wagner and Dray, 2015).

2.4.4. Functional annotation of the potentially selected loci

For all the candidate SNP loci identified by the genotype-environment association analysis, we performed a BLAST search and annotation of their flanking sequences against the NCBI nucleotide database using the BLASTn option with the default settings, and also against the whole-genome sequence of the *Drosophila melanogaster* fruit fly (https://www.ncbi.nlm.nih.gov/genome/?term=drosophila+melanogaster).

3. Results

3.1. Pesticide detection

Traces of pesticides above detection limits, ranging from 0.32 to 7.49 ng/g, were found only in sediment samples from 10 of the 30 study sites (Fig. 1, Table 1, Appendix 3), consisting in all the sites from the Río Rapel tributary, two sites from the Río Mostazal tributary and two sites downstream the main river body. All three water samplings, including one during a rain-induced pulse of runoff, failed to detect the presence of pesticides along the Limarí river basin. The main compounds found in our study were the herbicide atrazine and its DEA metabolite, which were detected in nine sites (Table 1). The presence of insecticides was also revealed in four sites, three of which with carbofuran (0.72–4.01 ng/g) and one with permethrin (1.01 ng/g). Only three sites displayed the joint presence of herbicide and insecticide compounds (Table 1). In addition to the compounds found above detection limits, traces of three other pesticides, all below detection limits (Appendix 4), were observed in water and sediment samples. Traces of the atrazine metabolite DIA (Pulpica (#9): 0.011 μg/L) and the methidathion insecticide (Maiten 1 (#20): 0.010 μg/L; Ramadas 1 (#27): 0.014 μg/L) were found in water samples from the October 2016 sampling campaign, while traces of the tefluthrin insecticide were found in the sediment samples (Maiten 2 (#22): 0.79 ng/g; Ramadas 2 (#29): 0.51 ng/g). These traces, below detection limits, were

not considered in the statistical analysis.

3.2. SNP genotyping and outlier detection

The Illumina sequencing procedure generated around 1.44 million reads per individual. The original filtering steps conducted by the SNPsaurus (LLC) staff produced 3817 SNPs (Table 2). By removing individuals with more than 40% missing data, we retained a total of 551 samples (see Appendix 6 for post-filtering sample sizes per sampling location). With additional, more stringent, filtering steps we removed, among others, 259 loci that departed significantly from Hardy-Weinberg equilibrium in more than 30% of the sampling locations and 98 loci that were linked to others with a correlation higher than 0.7, leading to a final dataset of 2056 high-quality SNPs (Table 2), with a mean depth value both per locus and per individual of 45.2 reads. At the population level, relatively few loci significantly deviated from Hardy-Weinberg equilibrium, with an average of 8.9% of the loci at an $\alpha = 0.05$ significance level, which dropped to 0.4% after sequential Bonferroni corrections.

Regarding the outlier identification, we recognized 291 oultiers using four different approaches (Table 2). Bayescan detected 9 outliers at a 5% FDR. Based on the scree plot of the PCA conducted in pcadapt, the first three components were retained, and 82 SNPs were identified as outliers with a 10% FDR. The spatial AEM and MEM frameworks allowed the detection of 111 and 100 outliers, respectively. Only 11 outlier SNPs were detected by more than one method. All the outliers and their corresponding detection method are listed in Appendix 7. To produce a neutral SNP dataset, we removed these outlier loci from the total dataset, obtaining a subset of 1765 putatively neutral SNPs (Table 2).

3.3. Population genetic diversity and differentiation

Globally, our SNP markers revealed relatively low levels of genetic diversity and very low population differentiation in the mayfly *Andesiops torrens* along the Limarí watershed. No marked difference in heterozygosity or allelic richness were found between the neutral and the total SNP datasets (Appendix 6). Expected heterozygosity (mean $He = 0.25$) appeared slightly higher than observed heterozygosity (mean $Ho = 0.22$). Observed heterozygosity was the parameter showing the most variation between sampling sites, ranging from 0.195 to 0.233 in the neutral dataset. Levels of population genetic differentiation were very low but more pronounced in the total than the neutral SNP dataset, with a mean pairwise F_{ST} of 0.0045 from the total dataset (max. value = 0.009) and 0.0039 from the neutral dataset (max. value = 0.009) (Appendix 8 and 9). The global G_{ST} index across all populations was also low with a value of 0.033 in both datasets. Despite these low levels of genetic differentiation, the DAPC analysis revealed that some populations were clearly differentiated (Fig. 2, see also Appendix 8 and 9). The population genetic structure suggested by this analysis (i.e. 20,5/12,13/21,29/15/18/28/all remaining sampling sites) was statistically significant, as indicated by an AMOVA (Analysis of Molecular Variance), with 9% of the genetic variance explained between these groups ($F_{CT} = 0.0009$, $P = 0.018$) and 3% between populations within these groups ($F_{SC} = 0.0003$, $P = 0.226$). No pattern of isolation by distance was detected with Mantel tests, neither by considering Euclidian distances ($r = -0.004$, $P = 0.523$) nor hydrographic distances following the river network ($r = -0.080$, $P = 0.245$). The overall genetic differentiation was not explained by the hydrographic structure within the Limarí river basin, with less than 1% of the genetic variance distributed between rivers irrespective of the SNP dataset used ($F_{CT} \leq 0.0001$, $P > 0.05$).

No effects of pesticides were found on population genetic structure and diversity. The sites where pesticides were detected were not significantly differentiated from those where no such contamination was observed (AMOVA: $F_{CT} \leq 0.0002$, $P > 0.05$), considering the neutral and the complete SNP datasets previously described and even a dataset composed only of the 291 outliers. No significant differences were detected between the population levels of genetic diversity and differentiation between the two site groups (*t*-tests: $P > 0.05$ in all cases).

3.4. Analysis of locus-environment associations

The reduction of the number of variables by the CLV approach led to the selection of eight environmental variables (Appendix 10), which include seven water parameters (maximum water temperature, calcium, nitrate and iron concentrations, cumulated pesticide amount detected, mean water velocity and turbidity) and one habitat characteristic (mean stream depth during biological sampling). At an FDR of 10%, locus-environment associations were detected for three outlier SNPs: 9711.380.34 and 12149.55.43, originally identified using the AEM approach, and 15494.92.61 identified with the MEM approach (Table 3, Appendix 11). All these associations involved only one environmental variable: the onsite maximum amount of pesticide detected. All three associations were maintained after controlling for spatial autocorrelation. The Moran spectral randomization test was only performed for the association with SNP 9711.380.34, since significant spatial autocorrelation was only found for this outlier loci (Appendix 12) and also revealed a significant association (Moran spectral randomization *P*-value: 0.008).

3.5. Functional annotation of the putatively selected loci

The blast searches based on the sequences of the three SNPs against the Genbank database showed significant matches with insect DNA sequences (Table 4). Two SNPs have sequences similar to coding gene sequences: SNP 9711.380.34 matched a portion of the low-density lipoprotein receptor-related protein 2 (LRP2) transcript of the brown planthopper *Nilaparvata lugens* and SNP 12149.55.43 matched a portion of the dumpy transcript variant J of *Drosophila melanogaster*. The SNP 15494.92.61 showed the poorest match with a whole genome shotgun (WGS) sequence from the mosquito *Aedes albapictus*, but still with 78% sequence similarity. The blast against the *D. melanogaster* genome revealed that each SNP sequence match a region on a different chromosome.

4. Discussion

4.1. Pesticide detection in the upper Limarí watershed

We provide the first rigorous evidence of pesticide contamination in freshwaters from north-central Chile. While a temporal monitoring would be needed to appraise the full extent of such contamination for the Limarí watershed, our study shows many pesticide residues may be found in freshwater sediments. The measured concentrations are generally low, however the cumulative effect of some organophosphate insecticides, such as carbofuran and permethrin, may be quite significant.

Pesticides were detected at about one third of the study sites, indicating that their distribution warrants concern. This was particularly true for the Rapel tributary since the pesticides occurred at all sampling locations from this river section, despite the fact that our land use data do not suggest any greater agricultural pressure in this area relative to other sections of the watershed. The detected concentrations were slightly higher than those reported for other pesticides by Barra et al. (2001a, 2001b) in lakes

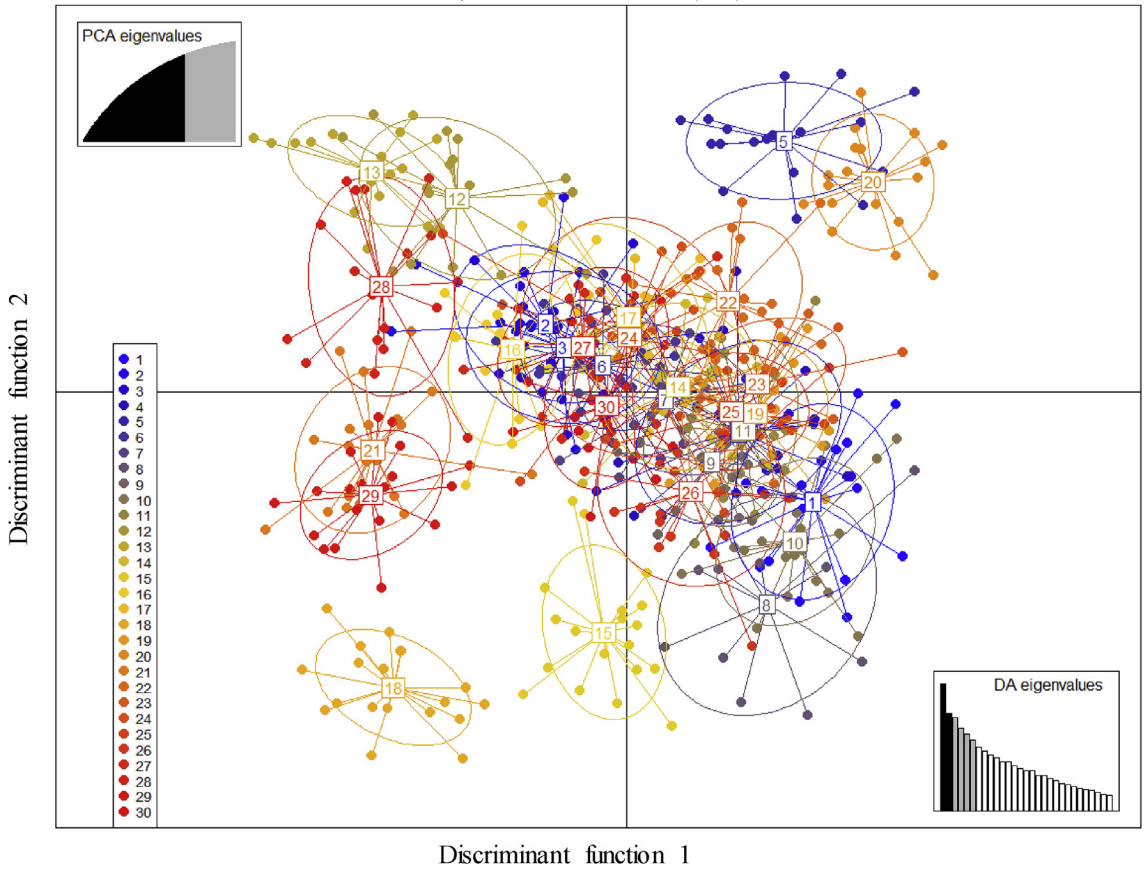

Fig. 2. Analysis of the population genetic structure of *Andesiops torrens* along the Limarí basin using discriminant analysis of principal components (DAPC) on the complete set of SNP loci (2056). Study site numbers (1–30) are as described in Table 1 and Fig. 1.

Table 3
Outlier detection method and Pearson correlations, *t*-test *P*-value and *Q*-value of the locus-environment associations identified with a false discovery rate of 10%. SNP sequences are provided in Appendix 11.

SNP	Outlier detection method	Environment association	*r*	*P*	*Q*
9711.380.34	AEM	Max. pesticide amount	−0.56	0.001	0.10
12149.55.43	AEM	Max. pesticide amount	0.60	<0.001	0.05
15494.92.61	MEM	Max. pesticide amount	0.61	<0.001	0.05

AEM: asymmetric eigenvector maps. MEM: Moran eigenvector maps.

Table 4
BLAST results and comparative genomics of the potentially selected SNP loci in *Andesiop torrens* populations of the Limarí basin. †: genome position determined by genome blast of the transcript of the best regular BLAST results containing the SNP sequences due to the lack of significant hit of the SNP sequence against the *D. melanogaster* genome; WGS: whole genome shotgun. SNP sequences are provided in Appendix 11.

SNP	BLAST results				Genome position
	Locus name - Source	Species	E-value/Match	Sequence ID	*Drosophila melanogaster*
9711.380.34	Low-density lipoprotein receptor-related protein 2. mRNA	*Nilaparvata lugens*	7.00E-09 51/57(89%)	XM_022341698.1	Chr. X† (9259911–9262683)
12149.55.43	Dumpy (dpy) - transcript variant J. mRNA	*Drosophila melanogaster*	9.00E-10 47/54(87%)	NM_001258947.3	Chr. 2L (4485645–4485698)
15494.92.61	Isolate C6/36 tig00000476. WGS sequence	*Aedes albapictus*	0.01 43/55(78%)	MNAF02000288.1	Chr. 3R (9244459–9244503)

from southern Chile, but lower than that described in central and south-central Chile (Baéz et al., 1996; Climent et al., 2018; Cooman et al., 2005; Retamal et al., 2013), where agriculture has been developed across much larger areas (Meza et al., 2012), and where elevated levels of pesticides, including atrazine as detected in the Limari watershed, are often reported in water samples.

When compared to other regions, it appears as though either leaching from nearby agricultural fields is more limited in the Limarí watershed or that pesticides are more readily degraded in this region. The high temperatures, sun exposure and low annual precipitation in this area could contribute to this, as they all can affect the environmental fate of pesticides in croplands (National Research Council, 1993). Furthermore, the highly sorptive capacity of dry mineral soils within arid regions (Shunthirasingham et al.,

2010) supports a compartmentalization of the pesticides to substrate rather than to water. This may particularly be the case for the three compounds detected in our study that tend to be hydrophobic ($2.12 < \text{Log } P < 6.5$; Benfenati et al., 2003). In the upper Limarí watershed, soils are characterized by fine-textured alfisols with subsoils rich in calcium carbonate (Casanova et al., 2013; Squeo et al., 2001). Pesticides could thus bind to soil particles in this section of the watershed and then be discharged from croplands into the river with the sediments after precipitations.

Atrazine and its DEA metabolite were the main compounds detected in our study, which corroborates the wide use of this herbicide across Chile (Baéz et al., 1996; Climent et al., 2018; Diez, 2010; Retamal et al., 2013). To our knowledge, this is the first time that permethrin (pyrethroid) and carbofuran (carbamate) were reported in a Chilean watershed, suggesting that these two insecticides may be more specifically applied in the Limarí or in watersheds from northern Chile. Both compounds are known to affect the aquatic fauna (Antwi and Reddy, 2015; Li et al., 2017; Münze et al., 2015; Rocha et al., 2018), including mayflies (Antwi and Reddy, 2015). Although the concentrations found in our study appear way below the threshold effect benchmark (TEB) proposed by Nowell et al. (2016) for benthic invertebrates (TEB_{int} Atrazine = 130 μg/g of Organic Carbon (OC), Carbofuran = 43 ng/g-OC, Permethrin = 420 ng/g-OC), they were expressed in ng/g of sediment dry weight (dw), rather than normalized to organic carbon (Amiard and Amiard-Triquet, 2015). The fraction of organic carbon in our samples was not quantified; however based on published data on the organic fraction in sediments from the study area (Astudillo, 2011; Copaja et al., 2014), we can extrapolate that the proportion of organic carbon in sediments of the study area should rarely exceed 5%. Under such conditions, our maximum concentration of carbofuran (4.01 ng/g-dw) could be higher than the TEB value for this compound (43 ng/g-OC) and much closer to its likely effect benchmark (LEB_{int} Carbofuran = 430 ng/g-OC; Nowell et al., 2016).

Carbofuran is highly toxic and its use is restricted in many countries (WHO, 2009). It remains authorized for use in Chile until September 2020 (http://www.sag.cl/ambitos-de-accion/plaguicidas-y-fertilizantes/78/registros), and its detection in river sediments is of note relative to both ecological and human health concerns. Its widespread use could contribute to the adverse effects of pesticide exposure that have been demonstrated on health of agricultural workers in the north-central region of Chile (Corral et al., 2017). The possible presence of two other highly toxic insecticides such as tefluthrin (pyrethroid) and methidathion (organophosphate), although found below method's detection limits, also raises concerns and calls for future assessments of their use and contamination impact. Given that the estimates of pesticide contamination along this section of the Limarí watershed might be underestimating the true exposure risks, we can conclude from our study that the aquatic biota may be at moderate risk locally, which tends to be corroborated by our population genetics data.

4.2. Pesticide contamination drives adaptive but not neutral genetic variation in A. torrens

In theory, environmental contaminants can affect all the micro-evolutionary forces that govern genetic distribution (Brady et al., 2017; Oziolor et al., 2016; Ribeiro and Lopes, 2013): they can cause genetic drift, alter gene flow, increase mutation rates and drive local adaptations. The effects on genetic patterns ultimately depend on the interplay between these forces. Here, we revealed a moderate level of genetic diversity in populations of *A. torrens* across the study area that is comparable to levels described in other mayfly species with RADseq data (Polato et al., 2017). We found no signs that pesticide exposure led to decreases in genetic diversity in *A. torrens* nor that it had a population structuring effect. While genetic erosion is a possible outcome of contaminant exposure (van Straalen and Timmermans, 2002), there is no consistent evidence regarding the effects of environmental contamination on genetic variability, with increases (Lado-Insua et al., 2011; Stambuk et al., 2013), decreases (Benton et al., 2002; Forfert et al., 2017; Fratini et al., 2008; Krane et al., 1999) and no change (Breitwieser et al., 2018; Giska et al., 2015; Martins et al., 2009; Miller et al., 2012) being reported. The lack of evidence for genetic erosion in contaminated sites can indicate that pesticide exposure does not provoke genetic loss (e.g. drift) in *A. torrens*, or that it is being compensated by enhanced mutation load due to genotoxic effects (Matson et al., 2006) or high gene flow between populations. Since we used di-allelic SNPs, we could not investigate whether mutation loads were increased in contaminated populations by analyzing the rates of rare alleles as done by Inostroza et al. (2016). Yet, our results support the hypothesis that high gene flow is occurring across populations and may thus compensate genetic erosion. Neutral genetic divergence across the study area was low and not influenced by geographic distance, revealing a well-connected metapopulation in the study area. This is consistent with previous reports of low levels of genetic differentiation at larger spatial scales, including across basins (Sabando et al., 2011), indicating that *A. torrens* can readily disperse over significant distances. We did not detect evidence for pollution-mediated genetic differentiation, which also suggests that genetic exchange is occurring between contaminated and non-contaminated sites. A similar situation was described by Durrant et al. (2011) in the brown trout in the River Hayle (UK) contaminated by heavy metals. Connectivity between contaminated and non-contaminated sites can be important from an evolutionary perspective since it can improve the resilience of the populations to new disturbances as it raises effective population sizes and the potential for rapid adaptive evolution by maintaining high levels of standing genetic variation and spread of adapted genotypes (Barrett and Schluter, 2008).

Signatures of local adaptation to pesticide contamination were detected at three SNP loci. All of them correlate solely with local pesticide concentrations. While the detection of locus-environment correlations is no absolute proof of local adaptation (Dalongeville et al., 2018), these results strongly suggest that pesticide exposure is a strong selective factor for *A. torrens* that imposes pressures that overwhelm the homogenizing effects of high gene flow, a necessary condition for locally adapted genotypes to evolve under such circumstances (Kawecki and Ebert, 2004; Richardson et al., 2014). Such phenomenon is often reported in aquatic organisms (Al-Breiki et al., 2018; Diopere et al., 2017; Gonzalo-Turpin and Hazard, 2009; Nielsen et al., 2009; Sarup et al., 2009; Wang et al., 2013).

The putatively adaptive SNPs suggest that cuticle-related resistance mechanisms are involved in *A. torrens* in response to pesticide contamination. Two of them, actually, belong to functional genes, LRP2 and Dumpy, which are implicated in cuticle-related processes. Cuticle can play a role in resistance to insecticides in insects (Bass and Jones, 2016; Benoit et al., 2016; Seixas et al., 2017). Thus, these two genes make plausible candidates for pesticide resistance in *A. torrens*. LRP2, also called Megalin, is a large protein that is broadly expressed in epithelial tissues and mediates endocytosis of a wide range of ligands from the apical surface (Moestrup and Verroust, 2001). It is involved in cuticle melanization and suspected to have multiple roles in regulating cuticle assembly (Riedel et al., 2011). Megalin-mediated endocytosis is also one of the mechanisms conferring resistance to the α-amanitin toxin in

Drosophila melanogaster (Mitchell et al., 2017). Dumpy is a very large extracellular protein required to maintain tension at epidermal-cuticle attachment sites (Wilkin et al., 2000). A dumpy-like gene, among others, was found differentially expressed between pesticide-resistant and pesticide-susceptible strains of the bed bug *Cimex lectularius* (Mamidala et al., 2012).

It is difficult from our study to clearly evaluate the fitness cost of these adaptive responses on the mayfly individuals. Nevertheless, a plethora of fitness effects associated with pesticide resistance have been described in insects, on traits like fecundity, fertility, larval development and body mass in the case of pyrethroids (Kliot and Ghanim, 2012), which are generally explained by increased production of metabolic enzymes (Panini et al., 2016). In freshwater invertebrates, negative effects of pesticide exposure on emergence time (Du et al., 2013) and growth rate (Maul et al., 2008) have been reported. As the impact on such traits can clearly be linked to cuticle-related processes since the cuticle is involved in the molting process (Parle et al., 2017), from which these traits depend on, we can hypothesize that the mayfly species *A. torrens* may be affected similarly within the Limarí watershed. Another mechanism suggested by our study that can have important consequences on individual fitness is cuticle melanization. Melanization plays an important role in several physiological processes in insects, such as immunity and wound healing (Nakhleh et al., 2017; Parle et al., 2017). Thus, any negative impact on cuticle melanization should have a direct fitness cost on *A. torrens* specimens, with potential cascading effects on reproductive and growth traits such as those described above. Although a direct assessment of these fitness costs is required, our study strongly suggests that there is a potential risk on *A. torrens* populations within this watershed. Future research is needed to confirm local adaptation and the potential candidate genes revealed by population genomic studies (Brodie et al., 2016; Dalongeville et al., 2018), with for example genome sequencing, transcriptomic analyses and common garden experiments, which will be facilitated by the ongoing genome sequencing of the mayfly *Ephemera danica* (https://www.hgsc.bcm.edu/arthropods/mayfly-genome-project).

5. Conclusion

Two main conclusions can be drawn from our study: i) pesticide contamination is occurring along the upper part of the Limarí watershed in north-central Chile, and ii) it has an impact on the endemic mayfly species *Andesiops torrens* at the population genetic level. Although the arid nature of the study area, and the subsequent reduced nature of the fate and transport of pesticides may have rendered their detection in riverine waters difficult, we brought evidence that the extent of pesticide contamination was relatively important along this section of the watershed. By detecting two potent insecticides never described in Chilean rivers, and potentially two others, with known effects on freshwater invertebrates (Antwi and Reddy, 2015; Rocha et al., 2018), we revealed the existence of a potential risk for the aquatic fauna in this region of the world, and also that pesticide contamination may be more extended than what we found in our study. This clearly shows that more efforts are needed to better assess the extent of pesticide contamination along this watershed, which belongs to a region where pesticide exposure is affecting the health condition of agricultural workers (Corral et al., 2017). It also stresses the need to evaluate the environmental impact of pesticide use along the watersheds of northern Chile, where agriculture is an important economic activity (Aitken et al., 2016), and yet any pesticide monitoring is lacking to date.

The population genomics approach that we used to detect some effects of the observed contamination on the mayfly *A. torrens* proved useful. By detecting several genomic regions potentially implicated in pesticide resistance and a possible resistance mechanism involving the cuticle, our study provides valuable information to investigate local adaptation to pesticides in insect species and highlights the great potential of RAD-seq data in aquatic ecotoxicology (Laporte et al., 2016). We found significant signals of natural selection at three loci despite potentially high levels of gene flow between populations, which indicates that the selective pressures by pesticide contamination on *A. torrens* must be strong. On the other hand, our results also suggest that this species may hold great resilience to future pesticide-contamination episodes in the Limarí watershed as local adaptation in well-connected metapopulations can facilitate the spread of adaptive alleles to newly altered habitats, and can, as such, ensure their persistence (Barrett and Schluter, 2008). Therefore, we revealed that *A. torrens* is sensitive to pesticide exposure but may hold great resilience to contamination episodes due to potentially high gene flow, corroborating that it is an ideal model organism to study evolutionary responses induced by pesticides on non-target, endemic species.

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Appendix A. Supplementary data

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