Temporal Genetic Dynamics among Mosquitofish *(Gambusia holbrooki)* Populations in Invaded Watersheds

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Running head: Temporal Genetic Dynamics of Invasive Mosquitofish

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Summary

The temporal components of genetic diversity and geographical structure of invasive mosquitofish populations are poorly known. Through the genetic monitoring of four consecutive cohorts of *Gambusia holbrooki* from three different river basins we aimed to determine temporal patterns of regional genetic variation and dispersal rates within invasive populations. Despite showing evidence of strong population size fluctuations, genetic diversity levels were maintained among local cohorts. We only detected temporal allele frequency changes associated with seasonal flooding that did not modify major trends on population structure among cohorts. Downstream gene flow coupled with increased connectivity at lowland locations to increase genetic diversity levels in these areas. A large proportion of local fish (up to 50%) were dispersers, often originated from locations within the same river basin. High dispersal capability, ecological tolerance, and reproductive traits likely promote river colonization. Finally, our results also confirmed that human-assisted translocations promote within and among basin gene flow and maintained levels of genetic diversity, particularly in upstream locations.

Introduction

Poeciliids are a small, live-bearing fish species from tropical and temperate American continental waters that have high ecological tolerance and that have successfully colonized new territories worldwide (Meffe et al., 1995). Some of them, such as mollies (*Poecilia latipinna*), guppies (*P. reticulata*), or swordtails and platies (*Xiphophorus spp*) are very popular for the aquarium trade, and now are distributed globally after invading natural habitats following escape from aquaria (Duggan et al., 2006). Others such as mosquitofish (*Gambusia holbrooki* and its sibling *G. affinis)* have been introduced worldwide as biological control agents for mosquito populations acting as malaria vectors (Krumholz, 1948; Pyke, 2005). Irrespective of the reasons for their introductions, invasive poeciliids have well-known negative impacts on native biota (Gamradt and Kats, 1996; Pyke et al., 2008; Stockwell and Henkanaththegedara, 2011). Several ecological factors contribute to the invasive success of poeciliid fish, including high thermal and salinity tolerance (Stockwell and Weeks, 1999), a short period for population recovery (Chapman and Warburton, 2006; Deacon et al., 2011), multiple paternity (Neff et al., 2008), and high dispersal capabilities (Rehage and Sih, 2004); moreover, rapid evolution in life-history traits has been reported in newly founded

populations of guppy *P. reticulata* (Reznick et al., 1990, 1997). Similarly, recently translocated populations of *G. affinis* from shared ancestors in Hawaii (Stearns, 1983) and North-America (Stockwell and Vinyard, 2000) displayed divergences among introduced populations, reflecting quick environmental adaptation (reviewed in Reznick and Ghalambor, 2001) that can result in diverse impacts on ecosystem functioning and structure (Bassar et al., 2010).

In Europe, mosquitofish, *G. holbrooki*, was introduced in 1921 when 12 specimens from North Carolina were released into a pond in SW Spain (Vidal et al. 2010). A year later, a few hundred individuals from their offspring were transplanted to Italy (reviewed in Navarro-Garcıa, 2013) and from there to every other European and Mediterranean country in the following years (Sholdt et al., 1972). Introduced mosquitofish are now playing a key role in Spanish Mediterranean freshwater ecosystems by competing and displacing endemic fish species such as the Spanish toothcarp, *Aphanius iberus*, and the Valencia toothcarp, *Valencia hispanica* (Rincon et al., 2002; Alcaraz et al., 2008; Carmona-Catot et al., 2013). The reduced genetic variation in mtDNA among European *G. holbrooki* populations supports the above-mentioned historical records confirming a common source for the majority of these populations and indicates a notorious uniformity among Iberian populations (Vidal et al., 2010). However, substantial genetic diversity at nuclear markers has been observed among Iberian populations from distinct river basins (Vidal et al., 2012; Sanz et al., 2013), as has variation in life-history traits such as size-at-maturity, reproductive effort, and gonadal size (Benejam et al., 2009; Carmona-Catot et al., 2011). Reduced mitochondrial variation and significant nuclear divergence was also observed among introduced populations of the guppy in Australia, and yet substantial adaptive variation was probably conserved in these populations after founder effects related with the introductions (Lindholm et al., 2005). More recently, Deacon et al. (2011) have shown that guppy populations founded from a single gravid female can preserve enough adaptive potential to colonize new territories. Compared to the Australian ones, European mosquitofish populations apparently retained most of the genetic variation of their American sources (Sanz et al., 2013). In fact, these American sources are located in areas colonized after the last glacial retreat, and the invasive potential of mosquitofish was probably already acquired in these populations as a response to population stress during the post-glacial northward expansion (Díez-del-Molino et al., 2013).

While the geographic patterns of genetic diversity among populations of mosquitofish have been studied extensively both in the original American basins (Wooten et al., 1988; Scribner

and Avise, 1993; Hernandez-Martich and Smith, 1997) and in invaded areas (Ayres et al., 2010, 2013; Purcell et al., 2012; Vidal et al., 2012; Sanz et al., 2013), the analysis of the temporal stability of the divergences among populations has received less attention. In the Everglades, a pronounced dry-down event resulted in the spatial reorganization of the population structure of mosquitofish between 1996 and 1999. In that short period of time, significant genetic changes arose at some locations due to extinction and recolonization from neighbouring populations as a response to water level fluctuations (McElroy et al., 2011). In contrast, McClenaghan et al. (1985) observed stable temporal genetic composition of mosquitofish populations within the Savanah River drainage, except a single pond affected by thermal effluents where sometimes temperatures reach 50ºC. In fact, mosquitofish quickly adapt in response to environmental factors such as salinity and thermal gradients (Congdon, 1994; Meffe et al., 1995; Purcell et al., 2012), pollutants (Tatara, 1999; Tatara et al., 2002), or the presence of predators (Langerhans et al., 2007). Some of the above adaptations were maintained over time even in the absence of barriers to gene flow (Purcell et al., 2012).

Rapid adaptive responses on mosquitofish are relevant in a context in which climate change most likely will stress extreme conditions in the Mediterranean areas of Europe (Giblein and Deque, 2003; Parmesan and Yohe, 2003; Ormerod, 2009). This will probably favour the establishment, spread, and invasive success of adaptable species with broad environmental tolerances, short generation times, and high rates of dispersal such as the *Poeciliids* (Deacon et al., 2011). Díez-del-Molino et al. (2013) described significant genetic divergence of Spanish mosquitofish populations at regional scale both within and among basins, but their study lacked of the temporal resolution needed to validate factors potentially involved in population divergences as the hydrological regimes or seasonal variation in levels of gene flow. Because Mediterranean streams are commonly shaped by irregular hydrological regimes, including periodical severe droughts and floods (Serrano et al., 1999; Trigo et al., 2004), we hypothesized the genetic diversity patterns, structure and levels of gene flow to be variable among seasons and generations in Spanish populations of mosquitofish.

Microsatellite loci have been recently regarded as limited when used for population genetic analyses, pointing out that some of their properties (i.e. rate of mutation) if not fully understood, may confound inferences (Putman and Carbone, 2014). However, several studies have shown that microsatellites perform similarly or better when compared with the same

number of other makers, such as Single Nucleotide Polymorphisms (SNPs), for revealing fine-scale processes (Ross et al. 2014; Defaveri et al. 2013). Hence, to date, microsatellite markers remain as one of the most cost-effective and widely used markers for population genetic inferences. In this work we used microsatellite markers to analyze patterns of genetic diversity among four consecutive *G. holbrooki* cohorts in invaded locations of three Mediterranean basins in NE Spain. Contrary to single time-point surveys, this temporal sampling provides temporal resolution and a wider perspective of the dynamics of gene flow and genetic diversity variation in invasive species. Such information may lead to a better understanding of the population genetics dynamic of local loss/recovery of genetic diversity following invasions and after periodical floods and droughts, as well as the dispersal patterns

contributing to the species' invasive success.

Methods

Sample collections

Fish were collected in two consecutive years (2010 and 2011) at several sites distributed along three river watersheds in NE Spain: the Muga, Fluvià and Ter rivers. The Ter River is the largest, with a basin area of 2955 km^2 and headwaters in the Pyrenees. The Fluvià (974 km^2) and Muga (758 km^2) are typical coastal Mediterranean streams (further details in Alcaraz and García-Berthou, 2007). All three rivers are subject to a Mediterranean climate, with severe summer droughts and autumn floods (Trigo et al., 2004). Water flow during the study period was analyzed from data collected at the respective gauging stations closest to the river mouth.

Mosquitofish are currently absent from the upper course of these watersheds; hence, we collected samples from the middle and lower courses of the three rivers, located at a maximum of 50 km from the mouth. We selected eight locations (VM, CE, EP, BL, BA, PP, OY and CL) representing both different stretches of the three study basins (Table 1, Figure 1) and the four genetic clusters revealed from the analyses conducted on a larger survey of the 2010 adult fish cohort (Díez-del-Molino et al., 2013). These genetic clusters were primarily associated with river networks: the Muga cluster, Fluvià cluster, Ter cluster, and a tributary of the Ter River, the Onyar cluster. The 2010 analyses included 3 additional locations (MF, BY and TV) from which enough immature fish was available.

A total of 1,285 specimens of *G. holbrooki* were collected with a dip net from the riverbank. Sampling sites had similar habitat features, shallow areas (< 1.5 m depth) with relatively low water velocity and always well vegetated. Samples were collected at the end of summer (September) when an adult cohort mostly composed of fish born at the beginning of the spawning season in spring (April-May) was coexisting with its immature offspring born in August (Fernández-Delgado et al., 1997; Cabral, 1999; Perez-Bote and Lopez, 2005). The majority of the summer adult fish die shortly after summer. Thus, at the beginning of the following spawning season the reproductive group will basically be composed of overwinter mosquitofish born during the previous summer. Collected individuals were stored in alcohol 96% until they were processed in laboratory for DNA extraction. For each location and

cohort, up to 40 individuals were analyzed depending on fish availability (Table 1). Collected fish not displaying gonopodium and smaller than 20 mm were classified as immature, and to ensure that the captured adult fish in a population belonged to a single cohort, only females with a larger standard length than 25 mm and males larger than 20 mm were kept as adults (Carmona-Catot et al., 2011). Thus, the G_1 and G_3 cohorts were composed of sampled adult fish, and the G_2 and G_4 resulted from sampled immature fish. Unfortunately no immature fish was kept at BA location from the 2010 survey. Altogether, our sampling schema was composed of four consecutive cohorts, G_1 (born in spring 2010), G_2 (born in summer 2010), G_3 (born in spring 2011), and G_4 (born in summer 2011).

DNA extraction and microsatellite analyses

Genomic DNA was isolated from the caudal muscle of each individual by using the Real Pure DNA extraction toolkit (Durviz S.L., Paterna, Spain) and following the manufacturer's instructions. DNA was stored at -20C until further use in Polymerase Chain Reactions (PCRs). Genetic variation among individuals was analyzed at 11 previously identified microsatellite loci (Pooc-G_{49}, Mf13, Gafμ3, Gafμ5, Gafμ6, Gafμ7, Gaaf7, Gaaf9, Gaaf10, Gaaf13, and Gaaf15) that were amplified in two multiplex PCRs with forward primers fluorescently labelled (see Díez-del-Molino et al., 2013). Genotype peaks were resolved on a 3130 Genetic Analyzer using the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

Gene diversity within locations and genetic stability

For each locus, cohort conformation with genotype expectations under Hardy Weinberg equilibrium (HWE) was tested with the exact probability test implemented in GENEPOP software (Rousset, 2008). MICROCHECKER software (Van Oosterhout et al., 2004) was applied to identify null alleles that might be responsible for the observed HWE deviations, and their frequencies were estimated using FREENA (Chapouis and Estoup, 2007). GENEPOP was also used to test for linkage disequilibria among loci. The significance of the results was corrected for multiple comparisons using the false discovery rate method (Benjamini and Hochberg, 1995). Genetic diversity within the studied cohorts was assessed in terms of expected heterozygosity (H_E) and allelic richness (A_R) using FSTAT software

(Goudet, 2001). Wilcoxon's signed-rank tests were carried out between cohorts at each location to tests for significant changes in genetic diversity. Effective population sizes (N_E) for each cohort at each study location were estimated using linkage disequilibrium between loci in the LDNE 1.31 program (Waples and Do, 2008). Because the N_E estimation is greatly affected by allele frequencies close to 0 and 1 (Waples and Do, 2008) and the minimum for local sample size was 20, N_E was calculated by removing rare alleles with a frequency lower than 0.05 to avoid introducing biases due to possible genotyping errors.

Allele frequency stability between cohorts at each location was assessed by a permutation test (1000 permutations) in FSTAT and summarized by pairwise F_{ST} values (Weir and Cockerham, 1984). Genetic differentiation among cohorts from all locations was depicted by a two-dimensional plot from the principal components analysis (PCA) of the allele frequencies matrix in GENALEX 6.4. A hierarchical analysis of molecular variance (AMOVAs) using the ARLEQUIN software 3.5 (Excoffier et al., 2005) indicated the relative relevance of allele frequency changes among cohorts within locations (F_{CL}) as compared with divergence among locations (F_{LT}).

Connectivity between locations

The connectivity between locations in the study region was analyzed using the assignment tests implemented in GENECLASS 2 (Piry et al., 2004). For the G_2, G_3 and G_4 cohorts, the Bayesian Rannala and Mountain (1997) method was used to assign individuals of a given cohort to the most likely source location of the previous cohort (G_2 fish assigned to G_1 locations, G_3 to G_2, and G_4 to G_3). Due to sampling differences and to facilitate comparisons between cohorts, the assignment results for each location were pooled into three categories: (1) local origin, (2) origin from other locations in the same river, and (3) origin from locations in other rivers. For each location/cohort, we used R (http://.r-project.org) and the exact probability test to compare the observed assignment distribution of these three categories with the expected distribution for 1,000 simulated individuals of local origin. To obtain the simulated individuals we used the sampled genotypes of the location in the preceding cohort and the HYBRIDLAB software (Nielsen et al., 2006). Because we had no immature fish available from the BA location in 2010 (Table 1), we created two simulated G_3

sets of 1,000 fish. For the first simulation, we used G_1 genotypes at this location to generate 1,000 adult G_2 fish to be used as parents of G_3 individuals. Because there was a significant contribution of the CL location to the cohort G_2 in all sampled locations of the Fluvià basin (see results), in the second simulation we used the first 900 above simulated G_2 BA fish plus 100 simulated G_2 fish at the CL location as parents. In addition, assignment results for G_2 and G_3 indicated high relevance of BY and TV locations as source populations at the intra- and inter-basin levels, but because these two locations were not sampled in 2011, putative G_3 reference genotypes for each of these two locations were simulated as follows: For the BY location, we generated a simulated G_3 population using as parents 900 of the simulated G_2 fish for BY and 100 of the simulated for OY, according to the G_2 estimated assignment proportions obtained by GENECLASS analyses in the BY location. The first 40 individuals out of the 1,000 simulated in the G_3 were incorporated in the G_3 file of source locations. In the TV location, analyses on the G_2 indicated the local origin of the fish. We then simulated 1,000 G_3 individuals using as parents the sampled G_2 genotypes of this locality, and again we incorporated the first 40 fish of the simulation to the file including G_3 sources.

Results

Water flow

The average water flow during the two years period was different among rivers. The Muga (average: 5.58 m^3/s, range 0.14 - 386.18) and Fluvià (average: 5.23 m^3/s, range 0.73 - 234.54) rivers had lower flow (Mann-Whitney U-test, $P < 0.001$) than the Ter River (average: 19.58 m^3/s, range 1.47 - 323.13). However, the three studied rivers displayed a similar hydrological pattern among them and between years (Figure 2). As is often detected in Mediterranean streams, the two annual wet periods resulted in high flow pulses during few spring and autumn days. In 2010, the main wet spring week occurred from May the 4th to 8th, with a flow peak of 99 m^3/s in the Muga River, 40 m^3/s in the Fluvià River, and 78 m^3/s in the Ter River. In autumn, strong rains occurred from October the 11th to 14th, with peaks of 176, 182, and 130 m^3/s respectively. In 2011, the spring rains fell earlier, from March the 13th to 19th, and resulted in flow peaks of 370, 234, and 188 m^3/s in the Muga, Fluvià and Ter River, respectively. We detected quite similar rain peaks in autumn at the Muga (386 m^3/s) and Ter (323 m^3/s) rivers, but lower at the Fluvià River (autumn peak: 234 m^3/s), and all of them were in November, a bit later than in 2010. Snow melting during May and June in the Pyrenean

headstreams of the Ter River maintained moderate water flows in spring in both years. As the Spanish mosquitofish spawning season extends from May to September, it is likely that water flow peaked prior spawning in both years (Figure 2).

Diversity within locations

We did not observe differences in genotype distributions between sexes in either of the adult cohorts G_1 and G_3. We therefore pooled fish of both sexes as a single adult sample at each location for those cohorts. Across the four cohorts, 16 out of 37 collection genotype comparisons deviated from Hardy-Weinberg expectations after correcting for multiple tests (Table 2). In all cases, deviations were related with positive F_{IS} values, suggesting heterozygote deficit. MICROCHECKER results pointed to the presence of null alleles as potential reason for these HWE departures, particularly those involving the loci Gafμ6 (average null allele frequency, q=0.077), Gaaf10 (q=0.066), and Gaaf15 (q=0.056). Removing these three loci, only 1 (MF location at cohort G1) out of 37 collections deviated from Hardy-Weinberg expectations. However, highly correlated genetic diversity values were obtained between the full data set and the genetic diversity calculated from the corrected allele frequencies calculated by FREENA (H_E vs H_E^{ENA}, Pearson's correlation index = 0.98, P <0.001). Tests for linkage disequilibria between loci at each cohort and population resulted in only 3 out of 440 significant pairwise comparisons. Estimates of effective population size (N_E) attained finite values for the four cohorts in the CE, EP, and CL locations. In the other eight study locations we obtained an infinite large value of effective size in at least one cohort. Only in location PP the estimated N_E suggested a large population size across the four consecutive cohorts, while in all the other locations the estimated N_E values indicated variation in population size at short time scales.

The BA location had the lowest diversity levels (A_R and H_E) of all analyzed cohorts, while the CE, EP, and CL locations accumulated the highest diversity estimates in a particular cohort (Table 2). In three locations (BL, OY, and CL), significant (P<0.05) allele frequency fluctuations between consecutive cohorts were detected through a permutation test. These significances involved comparisons between the mature 2011 (G_3) fish against the immature 2010 cohort (G_2). Nevertheless, according to Wilcoxon-signed-rank tests, these allele frequency changes did not result in significant changes in diversity levels (A_R and H_E). The Principal Component Analysis (PCA, Figure 3) showed local cohorts clustering very close in

all studied locations except BL, where the G_3 and G_4 cohorts (2011) were notoriously separated from the 2010 collections (G_1 and G_2). Globally, PCA analyses highlighted the relevance of by river basin structure separation rather than temporal fluctuations on the distribution of the species genetic diversity in these invaded basins. The locations in the Ter River (BY, CL, and TV) appear in the core of this regional structure with mosquitofish at PP location (Fluvià River) being genetically close to them. Upstream locations in the Fluvià River (MF, BA and BL) grouped together, as did all studied locations from the Muga River (EP, CE and VM). All these divergences were related to the first PCA factor (42.3% of variance explained), while the Onyar river sample, a tributary of the Ter River, was clearly distinguished from the rest along the second PCA factor (24.03% of explained variance). AMOVA analyses indicated that most of the gene diversity (83.2%) is shared among locations and cohorts, only the 2.6% of the genetic variation resulted from allele frequency changes among cohorts within locations ($F_{CL} = 0.031$), and the 14.2% among locations ($F_{LT} = 0.142$). However, both the F_{CL} and F_{LT} components were highly significant ($P<0.001$). As already observed in mosquitofish populations in the study region (Díez-del-Molino et al 2013), F_{ST} values using FSTAT were virtually identical to those estimated by FREENA ($F_{ST} = 0.159$ vs $F_{ST}^{ENA} = 0.157$, per loci Mann-Whitney U-test $P = 0.898$). Permutation tests did not demonstrate significant changes in the average regional allele richness (A_R), gene diversity (H_E), and geographical structure (F_{ST}) among cohorts (Table 3). Geographical patterns of diversity were also maintained among cohorts either considering a hydrographical model or a genetic model adjusted to the four clusters suggested by Díez-del-Molino et al. (2013).

Connectivity among locations

Expected proportions of assignment based on the simulated fish indicated that some local specimens could be assigned to foreign sources from the same (average 4.5%) or other river basins (average 5.8%), but the majority of individuals were correctly considered of local origin (average 90.7%). In 43 out of 78 comparisons, the observed assignment significantly departed from expectations, with a significant number of migrants from locations in the same or different basin (Table 4). Overall, departures from expected proportions pointed to a temporally unstable local pattern of mosquitofish immigration from other locations within the same basin (average 14.5%), and from other basins (average 17.2%). In cohort G_2, only two

out of 11 collections presumably had complete local origin, and in G_3, only the BA location - out of eight - had a local origin when the admixed G_2 was used as the reference population. Five out of eight locations showed a local origin in the G_4 cohort. We observed large proportions of immigrant fish from locations within the same basin in all locations of the Muga River, but lower proportions in the Fluvià and Ter rivers. Interestingly, significant contributions of fish from other river basins were observed in the lowland locations of the three rivers. Locations TV and CL in the lower part of the Ter River and PP in the lower Fluvià River often provided fish to the other river basins. Finally, the significant contribution of the BY fish of the Ter River to the BL location upstream in the Fluvià River detected in G_2 and G_3 were the most striking results.

Discussion

Origin and temporal stability of local diversity

Local levels of genetic diversity measured as H_E (range 0.34 - 0.49) were significantly higher (Mann-Whitney U test $P < 0.05$) in the study region than in the invaded territories of Australia (range 0.21 - 0.53, Ayres et al., 2010), but no significant differences in allele richness (A_R) were detected between these two invaded territories (2.22 vs. 2.42, Mann-Whitney U test $P > 0.05$). Nonetheless, the estimated diversity levels were lower (Mann-Whitney U test $P < 0.05$) than in the Ebro River basin located 300 km southward (range 0.48 - 0.54; Díez-del-Molino unpublished data) and other Iberian locations southward (range 0.46 - 0.58; Sanz et al., 2013). The levels of diversity observed in the study region were similar to those described in Italian locations (Sanz et al., 2013). Such a pattern of local diversity matches the expectations of a stepping-stone colonization process of the species from western Spanish locations to more eastern basins in the Mediterranean region according to historical records on mosquitofish introductions to Spain (Navarro-García, 2013) and Europe (Krumholz, 1948). This geographical pattern of successive reduction of local diversity according to sequential introductions was also detected during the colonization process of the sibling species *G. affinis* from Texas (US) to New Zealand (Purcell et al., 2012; Purcell and Stockwell, 2014).

Despite exhibiting considerable fluctuations in population size involving demographic growth and decline from very large estimates to very few specimens, we did not detect significant

changes in diversity levels among *G. holbrooki* cohorts in the study locations. Iberian mosquitofish populations grow fast in the spring and summer seasons, while their abundances decrease dramatically during winter (Fernández-Delgado, 1989; Cabral, 1999; Carmona-Catot et al., 2011). The low temperatures and short photoperiod during winters in temperate territories have been proposed to promote and intensify bottlenecks in mosquitofish populations (revised in Pyke, 2005). However, fluctuations in effective size do not seem to represent an obstacle for the species to retain genetic diversity levels over generations, probably because of reproductive strategies such as multiple paternity, female long-term sperm preservation, and abundant offspring allow them to maintain diversity levels even for cohorts mainly composed of overwintering individuals (Echelle et al., 1989; Zane et al., 1999; Spencer et al., 2000). Increased reproduction of survival fish and dispersal from neighbouring locations (Jordan et al., 1998; Baber et al., 2002) are surely involved in the rapid response of mosquitofish to strong population decline induced by droughts (Ruetz et al., 2005) or floodings (Chapman and Warburton, 2006). Similarly, reproductive traits favouring a quick recovery of population effective numbers such as multiple paternity and sperm storage, and admixtures from distinct populations mitigated population bottlenecks in

Trinidadian populations of the guppy, *P. reticulata* (Barson et al., 2009).

We detected significant allele frequency changes in comparisons between G_2 and G_3 in three locations (BL, OY, and CL). Reported flooding peaks in our study area occurred before the next spawning season. Therefore it is likely that the flooding in autumn 2010 and spring 2011 contributed to the G_2 dispersal as immature fish in autumn and adults in spring. Similarly, flooding promoted gene flow among populations of other species inhabiting comparable Mediterranean areas such as the Spanish toothcarp *Aphanius iberus* (García-Marín et al., 1991; Araguas et al., 2007). Mosquitofish populations in the Everglades (US) showed substantial modifications in local diversity and population structure in areas affected by drought periods (McElroy et al., 2011). We did not observed such a pattern. Unlike McElroy et al. (2011), no significant allele frequency changes in comparisons involving immature and adult fish collected at the same time (e.g., G_1 vs. G_2 or G_3 vs. G_4) were observed. Overall, the temporal component of the genetic diversity was lower than the river network structure component ($F_{CL} = 0.031$ vs. $F_{LT} = 0.142$), without significant alterations on the geographical pattern among cohorts.

Gene flow and connectivity

Despite of the high rates of individual exchange (up to 50% of migrants) we observed, regional fine-scale population structure over cohorts is maintained. There is evidence of reduced dispersal among introduced populations of mosquitofish in New Zealand (Purcell and Stockwell, 2014) and Australia (Ayres et al., 2013); however, both studies were conducted on a larger geographical scale and within-river comparisons were limited. American mosquitofish populations along river basins displayed positive spatial autocorrelation of allele frequencies between populations at hydrological distances of 6-150 km (Smith et al., 1989). In the study of the G_1 cohort, Díez-del-Molino et al. (2013) reported a pattern of isolation by distance only within the Fluvià River involving locations up to 30 km apart along the river but not among locations less than 16 km apart in the Muga and Ter basins. In our study, substantial immigration was especially evident in the Muga locations in every cohort, but it was also evident in the Fluvià locations and in the CL location of the Ter basin in the G_2 and G_3 cohorts.

Large and fluctuating rates of migration among locations suggested gene flow induced by

episodic flooding in American mosquitofish (Hernandez-Martich and Smith, 1997). Within the time frame of our study, we reported spring and autumn rains that resulted in sudden flow peaks overpassing one or two orders the magnitude the yearly average water flow of each basin. In addition, these flow peaks occurred after several dry months having brought the river flow to the annual minimums. Our results indicated increased diversity in the lowland EP, PP, and CL locations when compared with the respectively upstream VM, BL, and OY locations (A_R 3.426 vs. 2.834, Mann-Whitney U test $P < 0.01$; H_S 0.443 vs. 0.421, Mann-Whitney U test $P < 0.05$), a pattern consistent with downstream dispersal during flood episodes (Congdon, 1995; Hernandez-Martich and Smith, 1997). We also detected that the CL, PP, EP, and TV locations in the lowlands were often the source of fish collected in other basins (Table 4), confirming the high connectivity of mosquitofish populations between basins in the lowland plain (Díez-del-Molino et al., 2013). Such connectivity can also be responsible for increased local diversity and reduced divergence among locations in these lowland locations ($F_{ST} = 0.076$ among lowland locations vs. $F_{ST} = 0.211$ among upstream locations; Mann-Whitney U test $P < 0.001$). In fact, the lowland areas of the three river basins are located in an overall low altitude plain with a complex network of irrigation channels, marshlands and lagoons that interconnect particularly during floods (Serra Ruiz, 2006). Unrestricted dispersal during floods resulted in lower divergence among mosquitofish populations in the floodplain (Díez-del-Molino et al., 2013), as observed on the SE coast of the US (Hernandez-Martich and Smith, 1997) and in the Greater Melbourne Area in Australia (Ayres et al., 2010). Substantial dispersal of mosquitofish after flooding was also reported in the marshes and wetlands of Florida (Jordan et al., 1998), where the species was capable of dispersing between wetlands via surface water runoff during periods of heavy rainfall (Baber et al., 2002).

It is not easy to disentangle the relative contribution of downstream mosquitofish transportation during floods and higher connectivity opportunities to the increased amount of local genetic diversity in lowland locations. The assignments of the G_2 cohort fish indicated a significant contribution of upstream locations (32.5%) to the EP location in the Muga basin, and in the PP location of the Fluvià River (12.5%), but the PP location also received relevant contributions of fish originated in the lowlands of other basins (35%). Finally, in the lowland location of the Ter basin (CL), the results indicated that most of fish had local origin, and there was only a significant contribution of fish from the Fluvià River (PP location). This connectivity pattern changed in the G_3 and G_4 cohorts, suggesting altogether that while often

relevant, the relative contribution of downstream migration and lowland connectivity

between basins always varies.

The role of upstream locations

Dominant downstream migration during flooding should result in the extinction of isolated upstream populations following the drift paradox (Müller, 1954). On the other hand, flooding effects could be ameliorated by active upstream dispersal, utilization of refugia in streams, and the higher individual fitness experienced by lower densities in upstream populations (Humphries and Ruxton, 2002). Mosquitofish can disperse at rates greater than 800 m/day in unimpeded corridors (Alemadi and Jenkins, 2007), with females being better disperses than males and juveniles (Congdon, 1994). Results in Chapman and Warburton (2006) indicated a higher tendency of upstream dispersal of mosquitofish among stream ponds separated by few meters (2-20 m), and Díez-del-Molino et al. (2013) reported upstream gene flow in locations on the middle course of the Fluvià River 6 km apart. Our results indicate that invasive mosquitofish have a temporally sustained high dispersal potential within rivers, including in the upstream direction. This dispersal together with high ecological tolerance and maintenance of genetic diversity by multiple inseminated migrating females might contribute to the survival of the uppermost locations and the upstream colonization of Iberian river basins by the mosquitofish.

Additional human-assisted transport contributes to the expansion and success of invasive species (Kolbe et al., 2004). Long-range dispersal events detected for mosquitofish introduced to New Zealand and Australia most likely reflected intentional human-assisted translocations (Ayres et al., 2010; Purcell et al., 2012; Purcell and Stockwell, 2014). Following the same logic, significant gene flow from the PP location in the lowland of the Fluvià River to the OY location in the upstream of the Ter River were attributed to human-mediated translocations of adult fish from the G_1 cohort (Díez-del-Molino et al., 2013). Similarly, our results suggest presence of fish from BY and CL in the upper course of the Fluvià River (BL) in cohorts G_2 and G_3, probably indicating traces of past or contemporary human-assisted dispersal to upstream locations.

Conclusions

In the present study, mosquitofish genetic diversity and population structure at regional scales

resulted stable among generations of *G. holbrooki*, despite local changes in population sizes between cohorts (i.e., low number of overwintering individuals), and fluctuations in environmental conditions (i.e., seasonal floods). As observed elsewhere (Hernandez-Martich and Smith, 1997; Congdon 1995), mosquitofish lowland populations were able to maintain higher levels of diversity and displayed lower differentiation than those located upstream by receiving variable proportions of migration from both within and among river sources, particularly during seasonal floods.

Reproductive traits such as multiple insemination, sperm storage, and abundant offspring often result in few migrant invasive mosquitofish gravid females harbouring substantial diversity from their source populations (Díez-del-Molino et al 2013). Our results indicate that such traits can also help to mitigate bottlenecks resulting from overwinter population size declines, and likely restore diversity in upstream populations following the purge by prevalent downstream water flow.

It is expected that the climate change will ameliorate winter temperatures and produce more suitable habitats for mosquitofish in the upstream parts of the Iberian rivers (Buisson et al., 2008; Hellmann et al., 2008; Pullin et al., 2009). In the study region as well as in other Iberian rivers, mosquitofish populations are currently expanding (i.e. Oscoz et al., 2008), therefore the current uppermost populations represent the edge of the invasive process. Certainly, the high dispersal capability and habitat tolerance contribute to mosquitofish dispersers to localize new suitable habitats. Nevertheless we also detected that human-mediated translocations can be responsible for introduction and recurrent gene flow, especially to some upstream locations.

Finally, temporal genetic monitoring provides the resolution to detect population genetic events that have relevant effects on the invasive dynamics and, therefore, need to be specifically addressed in future control programs to prevent invaders from spreading.

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Table 1: Description of the study locations. m.a.s.l.: altitude in meters above sea level. Geographical coordinates: all longitudes are East, and latitudes North. N: sample sizes (females) for every generation. Unsampled cohorts are indicated with a dash symbol.

Basin	Location	m.a.s.l	Code	Coordinates	G_1	G_2	G_3	G_4
Muga	Vilanova de la Muga	39	VM	3°2'29.38", 42°16'49.86"	40 (20)	40	40 (20)	20
	Castellò d'Empúries	17	CE	3°4'16.16", 42°15'17.54"	40 (20)	40	40 (20)	20
	Empuriabrava	2	EP	3°7'26.78", 42°14'14.97"	40 (20)	40	40 (20)	20
Fluvià	Besalú	150	BL	2°44'9.01", 42°11'27.41"	40 (27)	40	40 (20)	20
	Báscara	65	BA	2°54'51.88", 42°9'49.76"	40 (20)	-	40 (20)	20
	Sant Miquel de Fluvià	28	MF	3°0'46.72", 42°9'56.04"	40 (20)	40	-	-
	Sant Pere Pescador	5	PP	3°4'18.02", 42°10'44.81"	27 (12)	40	40 (20)	20
Ter	Banyoles	172	BY	2°44'54.49", 42°7'7.317"	40 (20)	22	-	-
	Onyar	70	OY	2°49'48.00",41°58'25.53"	40 (22)	40	41 (20)	20
	Colomers	41	CL	2°59'8.99",42°4'58.51"	40 (20)	40	40 (20)	18
	Ter Vell	2	TV	3°11'43.51",42°2'42.84"	38 (34)	39	-	-

Table 2: Population diversity and differentiation among cohorts in the study river basins. A_R: Allele richness; H_E: expected heterozygosity; F_{IS}: departures from Hardy-Weinberg equilibrium; N_E: estimated effective population size; v.l.: very large; F_{ST}: divergence from preceding generation; *: P < 0.05

		Muga			Fluvià				Ter			
Cohort	Diversity	VM	CE	EP	BL	BA	MF	PP	BY	OY	CL	TV
G_1	A_R	2.22	2.47	2.4	2.02	1.78	2.13	2.36	2.01	2.15	2.37	2.11
	H_E	0.428	0.492	0.471	0.412	0.341	0.414	0.481	0.458	0.447	0.474	0.449
	F_{IS}	0.094*	0.173*	0.075	0.066	-0.015	0.042*	0.031*	0.03	0.042	0.094*	0.123*
	N_E	141.8	100	82.3	v.l.	v.l.	66.5	v.l.	40.2	v.l.	83.8	29.1
G_2	F_{ST}	0.014	0.003	0.003	0.001	-	0.006	0.009	0.011	0.005	0.026	0.001
	A_R	2.19	2.42	2.45	2.09	-	1.97	2.41	2.27	1.98	2.26	2.24
	H_E	0.436	0.46	0.481	0.422	-	0.39	0.449	0.487	0.457	0.474	0.458
	F_{IS}	0.137*	0.137*	0.136*	0.069*	-	0.065	0.127*	-0.062	0.051	0.084*	0.064*
	N_E	v.l.	116.3	28.7	516.6	-	v.l.	v.l.	v.l.	34.7	23.2	v.l.
G_3	F_{ST}	0.033	0.022	0.029	0.116*	-	-	0.012	-	0.030*	0.087*	-
	A_R	2.32	2.28	2.52	2.32	2.04	-	2.4	-	2.19	2.44	-
	H_E	0.451	0.454	0.491	0.491	0.431	-	0.478	-	0.474	0.483	-
	F_{IS}	0.064*	0.021	0.014	0.148*	0.144*	-	0.069	-	0.053	0.147*	-
	N_E	396.5	235.9	55.4	25.5	v.l.	-	167.7		58.4	51.9	-
G_4	F_{ST}	0.005	0.004	0.004	0.024	0.015	-	0.02	-	0.006	0.018	-
	A_R	2.06	2.52	2.19	2.05	1.85	-	2.49	-	1.96	2.2	-
	H_E	0.413	0.476	0.446	0.45	0.395	-	0.495	-	0.439	0.467	-
	F_{IS}	-0.054	0.187	0.055	-0.006	0.083	-	0.043	-	-0.011	0.051	-
	N_E	47.2	74.2	25.3	1533.3	30.7	-	v.l.	24.9	10.7	-	-

Table 3: Genetic diversity patterns of *G. holbrooki* in the study region at every analyzed cohort. A_R: average allele richness within locations, Hs: average diversity within locations, H_T: total diversity, and F_{ST} average differentiation among locations. Two AMOVA results are presented based on hydrographical structure (three river basins) and on the four genetic clusters suggested in Díez-del-Molino et al. (2013). SC and CT are the percentage of total diversity assigned to differences among locations within group and among groups, respectively. * P-value < 0.05.

	Diversity levels				Hydrographic structure		Genetic clusters structure	
Cohort	A_R	H_S	H_T	F_{ST}	SC	CT	SC	CT
G_1	3.037	0.449	0.535	0.165	10.5*	7.8*	10.5*	7.4*
G_2	3.116	0.458	0.542	0.157	10.2*	7.2*	10.1*	7.0*
G_3	3.226	0.477	0.573	0.169	9.7*	9.2*	8.6*	10.1*
G_4	3.149	0.46	0.562	0.182	12.3*	7.2*	11.8*	7.5*

Table 4: Assignment proportions (in per cent). Between parentheses, proportions of assignment of a simulated cohort (1,000 individuals) of local origin. *: $P < 0.05$. a: comparison with local origin originated from G_1 data; b: comparisons with local origin generated from a simulated admixed G_2 cohort; c: G_3 sources included simulated G_3 populations for BY and TV (see text).

Cohort	Location	Local origin	Other locations within basin	Other basins (proportion)	Other basins (locations)
G_2					
	Muga: VM	65.0 (81.4)*	27.5 (12.5)*	7.5 (6.6)	
	CE	37.5 (70.5)*	50.0 (23.2)*	12.5 (6.2)	
	EP	55.0 (80.7)*	32.5 (5.5)*	12.5 (13.8)	
	Fluvià: BL	85.0 (94.1)*	5.0 (4.2)	10.0 (1.7)*	BY, CL
	BA	-	-	-	
	MF	62.5 (80,9)*	22.5 (16.4)	15.0 (2.7)*	CL, TV
	PP	52.5 (83.4)*	12.5 (2.1)*	35.0 (14.5)*	EP, CL
	Ter: BY	72.7 (95.1)*	22.7 (2.9)*	4.6 (2.0)	
	OY	97.5 (95.9)	0.0 (0.3)	2.5 (3.8)	
	CL	60.0 (78.9)*	17.5 (10.4)	22.5 (10.7)*	PP
	TV	72.5 (81.4)	15.0 (6.5)	12.5 (12.1)	
	Averaged	69.6 (86.9)	15.0 (6.4)	15.4 (7.6)	
G_3					
	Muga: VM	47.5 (79.6)*	47.5 (17.8)*	5.0 (2.6)	
	CE	30.0 (68.4)*	45.0 (28.4)*	25.0 (3.2)*	TV, PP
	EP	47.5 (80.0)*	27.5 (10.3)*	25.0 (9.7)*	TV, PP
	Fluvià: BL	57.5 (95.2)*	5.0 (4.0)	37.5 (0.8)*	BY, CL
	BA[a]	65.0 (89.6)*	27.5 (10.2)*	7.5 (0.2)*	CL
	BA[b]	65.0 (75.1)	27.5 (20.4)	7.5 (4.5)	
	MF	-	-	-	
	PP	57.5 (82.8)*	12.5 (4.2)*	30.0 (13.0)*	CL, TV
	Ter: BY	-	-	-	
	OY	80.0 (97.6)*	5.0 (0.7)	10.0 (1.7)*	PP
	CL	45.0 (84.1)*	17.5 (8.5)	37.5 (7.4)*	PP
	TV	-	-	-	
	Averaged	62.5 (90.9)	11.3 (4.6)	23.8 (4.6)	
G_4[c]					
	Muga: VM	65.0 (78.9)	30.0 (18.2)	5.0 (2.9)	
	CE	50.0 (68.7)	50.0 (26.1)	0.0 (5.2)	
	EP	40.0 (70.5)*	40.0 (19.8)*	20.0 (9.7)	
	Fluvià: BL	95.0 (96.6)	5.0 (2.2)	0.0 (0.9)	
	BA	100.0 (96.6)	0.0 (2.4)	0.0 (1.0)	
	MF	-	-	-	
	PP	50.0 (81.1)*	5.0 (2.5)	45.0 (16.4)*	CL
	Ter: BY	-	-	-	
	OY	90.0 (97.2)	5.0 (1.8)	5.0 (1.0)	
	CL	50.0 (91.7)*	30.0 (3.1)*	20.0 (5.2)*	PP
	TV	-	-	-	
	Averaged	70.0 (94.5)	17.5 (2.5)	12.5 (3.1)	

Figure captions

Fig.1: Geographical location of the collection sites. Black dots represent sites of collection of *G. holbrooki*. Location codes are presented in Table 1.

Fig.2: Water flow during the study period from data collected at the respective gauging stations closest to the river mouth. Each of the three basins of the study area are depicted in different colours. Dotted lines indicate the annual spawning period of the Iberian *G. holbrooki* populations according to literature (see text).

Fig.3: Principal component analysis (PCA) depicting the relationships among the studied *G. holbrooki* cohorts. Samples are projected onto the plane formed by the first two principal factors. Each dot represents a location and a cohort. Location codes are presented in Table 1, and cohorts are coded for every generation (G_1, G_2, G_3 and G_4). Different colours indicate the basin of precedence of the location.

Figure 1

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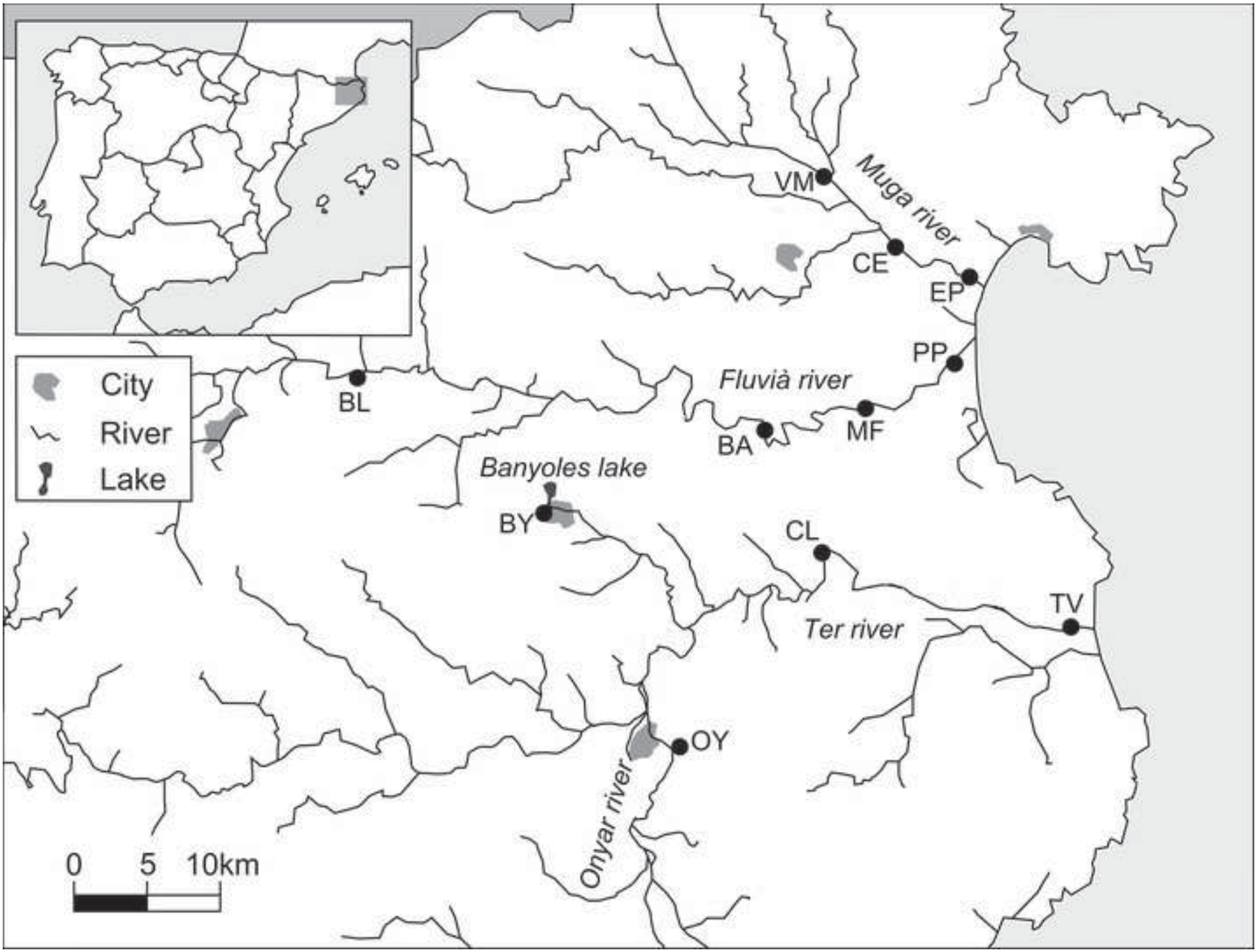

Figure 2

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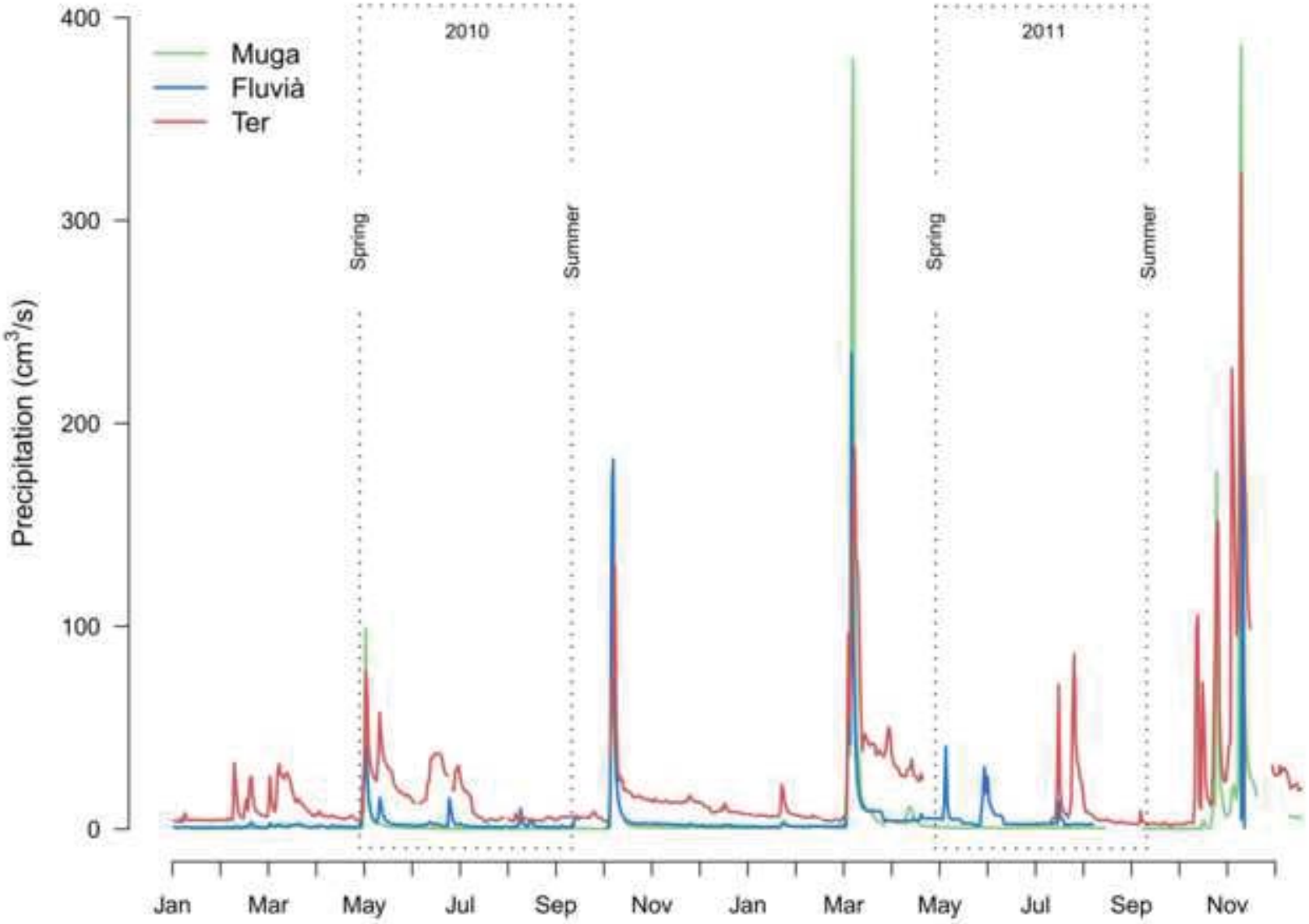

Figure 3

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