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1   **Functional responses of aquatic macroinvertebrates to flow regulation are shaped by**  
2   **natural flow intermittence in Mediterranean streams**

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4   **Running title (40 characters): Response to flow regulation and droughts**

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6   **Keywords**

7   Biological traits, functional diversity, functional redundancy, hydrologic alteration,  
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24      **Abstract**

- 25      1. Running waters in Mediterranean regions are strongly regulated by dams, which  
26      produce significant alterations to natural flow regimes. Climate change will  
27      reduce discharge and will increase flow intermittence in Mediterranean streams,  
28      which will lead to an intensified flow regulation to meet water demands. Very  
29      little is known about how the functional features of aquatic communities vary  
30      along combined anthropogenic flow alteration and natural intermittence  
31      gradients.
- 32      2. As intermittent streams are subjected to natural stress (droughts and flash  
33      floods), the flow regime alteration effect may differ from that observed in  
34      perennial rivers. Consequently, studies which aim to determine the effects of  
35      flow regulation on the functioning of aquatic communities in a global change  
36      context are crucial.
- 37      3. By applying Linear Mixed-Effect Models and null models to the  
38      macroinvertebrate communities from 65 stream sites in the Segura River Basin  
39      (SE Spain), we assessed the separate effects of natural flow intermittence and  
40      flow regulation, as well as their interaction, on biological traits and functional  
41      diversity indices.
- 42      4. Natural flow intermittence and flow regulation were mainly associated with loss  
43      of taxa with semivoltine or univoltine cycles and more sensitive aquatic stages  
44      (i.e., eggs), and with the replacement by taxa with multivoltine cycles and more  
45      resistant aquatic stages (i.e., adults). Flow regulation intensified the impact of  
46      natural flow intermittence on some biological traits, producing synergistic  
47      effects (i.e. decreasing interstitial taxa and tegument breathers and increasing  
48      taxa with aquatic adult stages). At the same time, antagonistic (life cycle) and

49 opposing (shredders) interaction effects were also observed. Functional diversity  
50 (FD), functional dispersion (FDis) and functional redundancy (FR) underwent a  
51 non-random decrease as the flow regime alteration increased, and a significant  
52 antagonistic interaction was also found between both stressors for FR.

53 5. In general, flow regulation effects were stronger in perennial than in intermittent  
54 streams because natural intermittence imposes a previous eco-evolutionary  
55 pressure on aquatic biota by selecting those resistant or recovery traits that  
56 confer resilience to anthropogenic flow regime alterations. Thus, the natural  
57 flow regime influences the functional sensibility of communities to  
58 anthropogenic flow alteration.

59 **1. Introduction**

60 Stream structure and functioning are substantially shaped by flow regimes, which  
61 influence the suitability of instream habitats (Benda *et al.*, 2004; Lytle & Poff, 2004)  
62 and their associated biological communities (Bunn & Arthington, 2002; Death, 2008).  
63 Anthropogenic flow regime alterations of natural flow regimes through water  
64 abstraction and dams involve changes in the intensity, frequency and seasonal  
65 predictability of floods and droughts. These changes can subsequently alter organic  
66 matter and sediment transport (Dewson *et al.*, 2007; Meador & Carlisle, 2012), with  
67 direct effects on riparian and aquatic biota, such as habitat homogenisation (Belmar *et*  
68 *al.*, 2013b), changes in indicator taxa and ecological traits (Guareschi *et al.*, 2014),  
69 reduced diversity (Bruno *et al.* 2016a; Lobera, 2017) and network simplification  
70 (Tonkin *et al.*, 2018). The ubiquity of flow alteration has led to a recent great interest in  
71 understanding aquatic ecosystem response to various types and degrees of flow regime  
72 alteration (see reviews by Dewson *et al.*, 2007 and Poff & Zimmerman, 2010). This is  
73 essential because changes in water availability and demands as a result of global change  
74 will intensify dam construction and flow regulation (Milly *et al.*, 2005; Chang *et al.*,  
75 2018), as well as increase the number of temporary rivers and the severity of flow  
76 intermittence (Larned *et al.*, 2010).

77 Mediterranean running waters are naturally subjected to flow extremes, which range  
78 from low to no flow during long dry seasons to flash floods during rainy periods (Gasith  
79 & Resh, 1999; Bonada *et al.*, 2006). In these aquatic ecosystems, flow variability and  
80 minimum flow are the major determinants of macroinvertebrate communities (Belmar *et*  
81 *al.*, 2013a), which are functionally important for numerous fluvial processes and  
82 services, such as biomass production, ecosystem engineering, nutrient cycling and  
83 resource processing (e.g. Covich *et al.*, 1999; Statzner, 2012). Intermittent rivers

84 support invertebrate species with a variety of physiological, behavioural, morphological  
85 and life-history adaptations to survive desiccation (Lytle & Poff, 2004; Datry *et al.*,  
86 2014, Stubbington *et al.*, 2017). The fauna of temporary watercourses exhibits traits  
87 related to resistance and recovery strategies, such as high growth rates, short lifespan,  
88 desiccation resistant stages and opportunistic/generalist feeding modes (Williams 2006,  
89 Bonada *et al.*, 2007b, Stubbington *et al.*, 2017), which confer resilience (*sensu* Hodgson  
90 *et al.*, 2015). Such traits may enhance their tolerance to similar environmental stressors,  
91 like those imposed by flow regulation. In fact, both natural intermittence and flow  
92 regulation affect water quantity and quality, habitat availability, as well as lateral,  
93 longitudinal and vertical connectivity (Bond *et al.*, 2008). These impacts can involve  
94 changes in aquatic communities (Mulholland *et al.*, 1997) and ecosystem functioning  
95 (Bruno *et al.*, 2016a).

96 Even though the isolated effects of flow intermittence (e.g., Bonada *et al.*, 2007b; Bond  
97 *et al.*, 2008; Belmar *et al.*, 2013b; Leigh *et al.*, 2017; Karaouzas *et al.*, 2018) and flow  
98 regulation (e.g. Poff & Zimmerman, 2010; Bruno et al. 2014; Ngor *et al.*, 2018) on the  
99 composition and structure of aquatic communities have been widely addressed, the  
100 understanding of how their interactions affect functional features is still limited. Studies  
101 in Mediterranean areas, aimed at determining the effects of flow regulation on the  
102 structure and functioning of stream communities and their interaction with natural flow  
103 intermittence, are essential to anticipate biological responses to stress intensification in  
104 a climate change context.

105 The intense development of trait-based approaches has improved the knowledge on the  
106 effects of anthropogenic impacts on ecosystem functioning over the last decade  
107 (Clapcott *et al.*, 2010; Laliberté *et al.*, 2010; Mouillot *et al.*, 2013; Gagic *et al.*, 2015).  
108 As the combination of species traits determines their likelihood to overcome

109 disturbances, a non-random taxa selection is expected along environmental gradients  
110 and after disturbances (Weiher *et al.*, 2011; Picazo *et al.*, 2012; Mouillot *et al.*, 2013).  
111 In addition, trait-based approaches allow functional diversity measures to be estimated  
112 (Laliberté & Legendre, 2010) at the community level, such as functional diversity  
113 (Tilman *et al.*, 1997), richness, evenness, divergence (Mason *et al.*, 2005, for a review)  
114 and redundancy (Rosenfeld, 2002). These indices reflect how an ecosystem operates in  
115 terms of niche differentiation, efficiency using resources, resistance and recovery,  
116 among others (Petchey & Gaston, 2006). Changes in functional traits caused by  
117 environmental stress could involve functional homogenisation (Clavel *et al.*, 2011),  
118 with reduced functional diversity (Sonnier *et al.*, 2014; Gutiérrez-Cánovas *et al.*, 2015)  
119 and loss of associated ecosystem goods and services (Buisson *et al.*, 2013).

120 Previous studies conducted in Mediterranean rivers have assessed functional changes in  
121 aquatic and riparian communities along natural gradients of water salinity and flow  
122 intermittence (Suárez *et al.*, 2017), as well as along anthropogenic stress gradients such  
123 as agriculture or flow regime alteration (Mellado-Díaz *et al.*, 2008, Gutiérrez-Cánovas  
124 *et al.*, 2015, Bruno *et al.*, 2016a,b). These studies found that functional diversity and  
125 redundancy measures decreased as environmental stress increased, regardless of  
126 whether the origin of such stress was natural or anthropogenic. Given that previous  
127 studies in aquatic communities have considered the biological effects of anthropogenic  
128 and natural stressors separately, unravelling their interaction effects is crucial to provide  
129 reliable measures of the impact of combined anthropogenic and natural stressors for  
130 management and conservation. On one side, such understanding could have  
131 implications to gain insight into the ecological consequences of the ongoing climate  
132 change in rivers by being able to anticipate biological changes in European freshwaters  
133 (Bonada *et al.*, 2007a), where climate change models predict increased aridity which

134 will lead to drought intensification and greater flow intermittence (IPCC, 2013). On the  
135 other, it constitutes a contribution to the urgent need to evaluate the consequences of the  
136 current Mediterranean and global increase in dam construction (Zarfl *et al.*, 2015).

137 Our main objective was to assess the effect of anthropogenic flow regime alteration,  
138 natural flow intermittence and their interaction on macroinvertebrate trait composition  
139 and functional diversity measures, at community level. In the most altered rivers, we  
140 expected similar changes to those caused by natural flow intermittence: dominant small-  
141 sized taxa, short cycles, multi-voltinism, aquatic adult stages, predators and diapause or  
142 dormancy resistance forms, together with lower functional diversity indices due to the  
143 functional simplification and homogenisation of aquatic macroinvertebrate  
144 communities. Given the resistance and recovery strategies developed by intermittent  
145 stream fauna to cope with flow cessation discussed earlier, we hypothesised that natural  
146 flow intermittence can moderate the impact of flow regulation. Thus, more antagonistic  
147 interaction effects could be expected and functional changes would be more intense in  
148 perennial than in intermittent rivers.

149 **2. Methods**

150 *2.1. Study area*

151 The Segura River Basin (18,870 km<sup>2</sup>, SE Spain) is an environmentally diverse basin due  
152 to human (alteration) and natural (climatic) gradients with a predominately semi-arid  
153 climate (see Bruno *et al.*, 2014). The catchment presents a strong climatic and  
154 altitudinal gradient from the northwest (NW) to the southeast (SE). Climate ranges from  
155 wet (>1,000 mm mean annual precipitation) and cold in the NW mountains (>1,000  
156 m.a.s.l.) to semiarid (<350 mm mean annual precipitation) and hot in the SE lowlands.  
157 This results in an average yearly precipitation around 362 mm (historical series:

158 1980/81-2005/06; <https://www.chsegura.es>). These conditions determine the existence  
159 of eight hydrological types (from large and perennial rivers to ephemeral streams), with  
160 differences in flow magnitude and intermittence (Belmar *et al.*, 2011). The basin has a  
161 high regulatory capacity (770 hm<sup>3</sup>, over 90% of natural input) provided by 24 dams and  
162 121 weirs, being one of the most regulated river network in Europe (CHS, 2008  
163 Grindlay *et al.*, 2009, 2011). Irrigation (responsible for 87% of water demands;  
164 Grindlay *et al.*, 2011) constitutes the main anthropogenic pressure on stream flows.  
165 There are two main flow alteration types in the Segura River Basin depending on the  
166 river type or natural flow regime (Belmar *et al.*, 2013b). Perennial rivers show sudden  
167 unpredictable water releases from big dams, which aim to provide water for irrigation in  
168 summer. However, natural temporary streams undergo drought intensification due to  
169 intense water diversions for agriculture from upstream dams. This is, therefore, a  
170 suitable area to study the biological and functional consequences of hydrological  
171 stressors in Mediterranean rivers and their interaction due to the variety of natural flow  
172 regimes and anthropogenic flow regime alterations.

173 Sixty-five freshwater stream reaches (conductivity < 5,000 µS cm<sup>-1</sup>) were selected in  
174 the basin to ensure large gradients of natural flow intermittence and regulation. The  
175 correlation between flow intermittence and hydrologic alteration was tested using  
176 Pearson correlation. Each reach was classified according to its degree of flow  
177 intermittence (Fig. 1; from “1” or most perennial to “8” or ephemeral; Belmar *et al.*,  
178 2011). Hydrologic alteration was assessed using the surface of irrigated land (%),  
179 number of dams (count) and regulatory capacity (hm<sup>3</sup>) in the drainage area of each  
180 sampling site (see Falcone *et al.*, 2010a,b and Belmar *et al.*, 2013b for details) as these  
181 variables are linked to the main flow regime alterations in the Segura Basin (Belmar *et*  
182 *al.*, 2010). Sites were assigned a score between 0 and 8 for each variable based on their

183 percentile value within the data range. The 1<sup>st</sup>, 20<sup>th</sup>, 40<sup>th</sup>, 60<sup>th</sup>, 80<sup>th</sup>, 90<sup>th</sup>, 95<sup>th</sup> and 98<sup>th</sup>  
184 percentiles were calculated in “non-zero” values and used as “disturbance thresholds” to  
185 assign the corresponding score for each site (Falcone *et al.*, 2010b; Belmar *et al.*,  
186 2013b). These irregular thresholds ensure the assignment of outstanding alteration  
187 values to the (scarce) most altered locations (Falcone *et al.*, 2010a). Finally, the three  
188 scores were summed, which provided a dimensionless index that potentially ranged  
189 from 0 (minimum flow alteration) to 24 (maximum flow alteration).

190 *2.2. Macroinvertebrate data*

191 Macroinvertebrates were sampled between 2010 and 2011 in dry-weather months (late  
192 spring and early summer). These months corresponded to the season of maximum  
193 aquatic invertebrate activity and environmental stress (droughts and flow regulation). A  
194 kick-net (500 µm mesh) was used following a multihabitat standardised protocol, with  
195 sampling effort proportional to each habitat occurrence (Jaímez-Cuellar *et al.*, 2002).  
196 The kick-sample contents were pooled into a unique site-sample and preserved in 96%  
197 ethanol. In the laboratory, organisms were identified at genus level, except for dipterans,  
198 which were identified at family, sub-family or tribe level in order to achieve a  
199 compromise between taxonomic resolution and classification effort. These taxonomic  
200 levels are frequently used to analyse trait composition and functional diversity measures  
201 (Dolédec *et al.*, 2000, Tachet *et al.*, 2010) due to the general lack of trait databases at  
202 species level. To perform analyses, the taxa Hirudinea, Oligochaeta, Ostracoda and  
203 Hydracarina were excluded, as trait information is unavailable or mostly incomplete at  
204 genus level (see Gayraud *et al.*, 2003; Tachet *et al.*, 2010).

205 The functional features of aquatic macroinvertebrates were characterised using 11  
206 biological traits and 63 trait categories (Tachet *et al.*, 2010). Traits included maximal  
207 potential size, life-cycle duration, potential reproductive cycles per year, aquatic stages,

208 reproduction, dispersal, resistance forms, respiration type, locomotion and substrate  
209 relation, food preference and feeding habits. These traits have been pointed out as being  
210 responsive to stress intensification in Mediterranean and temperate rivers (Bonada *et al.*,  
211 2007b). Each taxon was coded according to its affinity to each trait category using a  
212 fuzzy coding approach (Chevenet *et al.*, 1994). The fuzzy coded data were converted  
213 into percentages of affinity for each trait category. This procedure standardises the  
214 potential differences in the codification scores (i.e., different row sums for each taxon  
215 and trait).

216 *2.3 Functional diversity indices*

217 The community-level weighted means of trait values (CWM) were calculated to  
218 quantify the proportion of each trait category in each sample. “Taxon × traits” and  
219 “taxon × site” matrices were crossed to obtain the CWM matrix with the proportion of  
220 each trait character in each sampling site. Based on this trait information, a set of  
221 functional diversity indices was calculated: functional dispersion (FDis; *sensu* Mason *et*  
222 *al.*, 2005), functional evenness (FEve), functional richness (FRic), functional diversity  
223 (measured as Rao’s quadratic entropy; FD; Botta-Dukát, 2005) and functional  
224 redundancy (FR; Rosenfeld, 2002). Gower dissimilarity matrices (adapted for the fuzzy-  
225 coded traits, Pavoine *et al.*, 2009), using all the biological traits, allowed us to compute  
226 FDis, FEve and FRic. To calculate FD and FR, only effect traits were considered. Effect  
227 traits are those biological features that directly influence a specific function of the  
228 ecosystem (e.g. productivity, nutrient cycling) while response traits change according to  
229 the abiotic and biotic environment (e.g. climatic conditions and disturbance regime;  
230 *sensu* Díaz & Cabido 2001). We selected six effect traits (size, aquatic stage, dispersal,  
231 locomotion, food and feeding habits) based on the updated reviews done by Schmera *et*  
232 *al.* (2017) and Hevia *et al.* (2017). These traits are related to important processes

233 associated with ecosystem functions such as energy flow in food webs, nutrient cycling,  
234 aeration of sediments and recovery of disturbances (Covich *et al.*, 1999). For example,  
235 mobile animal species increase ecosystem resilience by connecting habitats and  
236 ecosystems through their movements (Sekercioglu, 2010).

237 FDis was measured as the abundance-weighted functional dispersion of the functional  
238 traits (i.e. functional  $\beta$ -diversity). To quantify this metric for each community, we  
239 estimated the weighted mean distance to the weighted community centroid (Laliberté &  
240 Legendre, 2010). FEve, which represents the regularity of the distribution and relative  
241 abundance of taxa in the functional space for a given community, was calculated by the  
242 method of the minimum spanning tree in a functional space based on an all-trait  
243 dissimilarity matrix (Villeger *et al.*, 2008). FRic was estimated as the hypervolume  
244 enclosing the functional space filled by the community (Villeger *et al.*, 2008). The  
245 number of axes retained to estimate hypervolumes was decided according to the method  
246 proposed in Maire *et al.*, (2015). This variable was standardised by its maximum, and  
247 ranged from 0 to 1. FD is an abundance-weighted metric that measures the mean  
248 pairwise dissimilarities of randomly selected taxa in a community as a way to describe  
249 the diversity of traits (Rao, 1982). Finally, given the variety of methodologies to  
250 estimate FR and the lack of consensus about them (Ricotta *et al.*, 2016), the approach  
251 that showed better performance in response to natural and anthropogenic disturbances in  
252 the study area was used (see Bruno *et al.*, 2016b). FR was obtained for each sampling  
253 site as the average number of taxa per functional group (Rosenfeld 2002; Laliberté *et*  
254 *al.*, 2010). Functional groups were considered sets of organisms with a suite of co-  
255 adapted characteristics and similar functional effects on the dominant ecosystem  
256 processes that, in comparison to other groups, present differences with clear ecological  
257 significance (Díaz & Cabido, 2001; Naiman *et al.*, 2005). Taxa were classified into

258 functional groups using a Ward's clustering method based on the effect-trait  
259 dissimilarity matrix. Ward's clustering method involves the use of Euclidean distances.  
260 Therefore, we ensured that the eigenvectors of a double-centred matrix obtained using a  
261 principal component analysis were positive, in order to verify that the Gower effect-trait  
262 dissimilarity matrix met this criterion (Laliberté & Legendre, 2010; Bruno *et al.*,  
263 2016b). We selected six functional groups based on distinctive functional effect traits  
264 through a Non Metric Multidimensional Scaling (NMDS) combined with an  
265 environmental fitting procedure done using trait characteristics instead of environmental  
266 variables.

267 *2.4 Functional responses to flow regime alteration and natural flow intermittence*

268 The individual and combined effects of flow regime alteration and natural flow  
269 intermittence on macroinvertebrate trait composition (defined by CWM) and functional  
270 indices were tested by Gaussian linear mixed-effect models (LME) as previously used  
271 in similar analyses (e.g., Bruno *et al.*, 2016b). The dependent variables were  
272 transformed (square root of arcsine, commonly used with proportions) to fulfil the  
273 assumptions of Gaussian models. LMEs included stressor slopes (flow alteration and  
274 natural flow intermittence) and the interaction between them as fixed terms, along with  
275 a random intercept to account for altitudinal (non-hydrological) variability. Such  
276 variability was considered a 6-level factor and represented different elevation ranges  
277 (from <200 to >1,000 m.a.s.l., each 200 m). LMEs produce two measures of goodness  
278 of fit, the marginal  $R^2$  associated with the fixed effects (those produced by hydrological  
279 stressors) and the conditional  $R^2$  that represents also the random effects (caused by  
280 environmental variation associated with elevation). These  $R^2$  are less susceptible to  
281 problems associated with mixed-effect models than common definitions of  $R^2$   
282 (Nakagawa & Schielzeth, 2013). We focused on the former as it is associated with the

283 effects produced by flow alteration and flow intermittence (fixed effects). LMEs were  
284 performed on functional indices by a backward-stepwise procedure to retain the model  
285 that minimised the Bayesian Information Criterion (BIC) in order to obtain the best  
286 model for each functional trait and functional index. As many tests had to be performed  
287 for the functional traits, the Benjamini & Hochberg (1995) correction was used to  
288 minimise the False Discovery Rate. Model assumptions were checked visually (residual  
289 normality and homoscedasticity).

290 In the best-fitting models, we distinguished additive effects when stressors did not  
291 interact, but were individually added to each other, and interactive effects when there  
292 was a significant interaction term. We followed the criteria proposed by Feld *et al.*  
293 (2016) for models obtained from trait-based data along stressor gradients. We  
294 considered that an interaction can be antagonistic, which means that the net effect of  
295 both is less than the sum of their independent effects, when the individual stressor  
296 coefficients take the same sign but the interaction coefficient takes the opposite sign;  
297 synergistic, when all the individual and interaction coefficients are of the same sign,  
298 thus the interaction effect is stronger than their additive individual effects; and  
299 opposing, if individual stressor coefficients have opposite signs no matter the sign of the  
300 interaction term's coefficient.

301 A relationship between functional indices and environmental stressors can be found  
302 simply because of the underlying taxonomic gradient (Villéger *et al.*, 2008). Thus null  
303 models were used to assess the non-randomness of the observed trends. For this  
304 purpose, we randomly reassigned traits to each taxon (999 runs) by re-examining their  
305 relationships with stressors and keeping the same trait combinations, richness gradient  
306 and taxon frequency of occurrence. For each simulation, we used the same model and  
307 procedure as for the empirical data. We examined the null model's statistical

308 significance using an exact two-tailed test to calculate the probability of the empirical  
309 value being significantly ( $\alpha = 0.05$ ) larger or smaller than the simulated distribution.

310 All the functional analyses were performed using the R software (R 3.4.0 version; R  
311 Core Team, 2017). Functional diversity indices (including the CWM matrix) were  
312 obtained using the function *dbFD* from the package FD (Laliberté & Legendre, 2010;  
313 Laliberté & Shipley, 2011). The LMEs were developed using the functions *lmer* and  
314 *lme* from the packages *lme4* (Bates *et al.*, 2015) and *nlme* (Pinheiro *et al.*, 2017),  
315 respectively. The NMDS was developed using the functions *metaMDS*, *envfit* and  
316 *s.class* from the *vegan* (Oksanen *et al.*, 2013) and *ade4* (Dray & Dufour, 2007)  
317 packages.

318 **3. Results**

319 3.1 Effects on trait composition

320 One hundred and twenty-four genera (plus 16 higher taxonomic levels) were identified  
321 in the study area (Table S1, Supplementary Material). The richest insect Orders were:  
322 Coleoptera (33%; 46 out of 140 taxa), Diptera (13%), Ephemeroptera (11%), Hemiptera  
323 (11%) Odonata (10%) and Trichoptera (10%). All the considered taxa were classified  
324 into six functional groups that represented different life strategies (mainly determined  
325 by body size, feeding habits, locomotion and substrate relationships) and ecosystem  
326 effects with a clear ecological significance on ecosystem functioning (Table 1; Fig. S1  
327 and Fig. S2, Supplementary Material).

328 Flow regime alteration and natural flow intermittence were weakly related. The  
329 correlation (Pearson  $r = -0.53$ ,  $p$ -value  $< 0.01$ ) was statistically significant but showed a  
330 moderate value. Such outcome was not enough to discard one of the stressors for  
331 analyses, as the correlation coefficient was out of the  $|0.70|$  threshold commonly used in

ecological studies (e.g. Zuur *et al.*, 2009; Dormann *et al.*, 2013). In addition, the absence of a non-linear relationship was verified (Fig. S3; Supplementary Material). Both stressors significantly increased the proportion of multivoltine taxa and reduced the proportion of univoltine and/or semivoltine ones (Fig. 2; Table S2, Supplementary Material), with an increase in the potential number of cycles per year. Besides, both stressors entailed a statistically significant reduction in the taxa with aquatic eggs and crawlers. Flow alteration also limited the presence of taxa with aquatic active dispersal. Meanwhile, the proportion of taxa with clutches in vegetation, diapause and/or dormancy as resistant forms, and full water swimmers increased significantly with flow intermittence, accompanied by a reduction in interstitial taxa. Natural flow intermittence also produced significant changes in food and feeding habits by increasing the proportion of taxa that preyed on invertebrates and vertebrates, but reducing the relevance of scrapers (Fig. 2; Table S2, Supplementary Material).

The interactions of both stressors produced antagonistic, synergistic and opposing effects on some trait categories (Table 2) as the effect of one stressor is conditional on the position along the gradient of the other stressor (Fig. 3). Antagonistic interactions were found in short and long life cycles, as proportions of taxa with shorter life cycles were higher in strongly regulated perennial rivers whereas long life cycles were more frequent in free-flowing perennial rivers or strongly regulated intermittent ones. Positive synergistic effects were found on the proportion of taxa with aquatic adult stages (maximum values in intermittent regulated rivers), but negative synergistic effects (minimum values in intermittent regulated rivers) on the interstitial taxa and tegument breathers (the interaction was significant and with the same sign as the individual coefficients; Table 2). Finally, flow alteration and flow intermittence interaction had an

356 opposing effect on shredder feeding habits, which decreased with flow alteration in  
357 perennial rivers but increased in intermittent streams.

358 3.2 Effects on the functional diversity indices

359 All the analysed functional indices, except FEve, significantly decreased with increasing  
360 flow alteration, whereas only FR decreased when flow intermittence increased,  
361 particularly in free-flowing rivers (Table 3, Fig. 3). The interaction of both stressors  
362 resulted in antagonistic effects on functional redundancy (FR) and functional richness  
363 (FRic), being the sign of the interaction term opposite to that of each stressor  
364 individually. Both indices responded similarly to disturbance, and were the most  
365 sensitive indices (the best models explained more than 30% of deviance). Conditional  
366 R<sup>2</sup> and marginal R<sup>2</sup> displayed similar values in the best-fitting mixed-effect models for  
367 all the functional indices. Thus, the elevation gradient had an inconspicuous influence  
368 on the spatial distribution of the functional indices compared with the effect of both  
369 stressors.

370 The null models revealed that stressors caused non-random changes in FDis, FD and  
371 FR, which were significant ( $p < 0.05$ ) for all the terms of the best-fitting models (Table  
372 4). However, null models did not reveal significant effects of the studied factors on  
373 FRic, which meant that the observed responses were related to the underlying  
374 taxonomic richness gradient.

375 **4. Discussion**

376 The use of trait-oriented functional approaches to evaluate the response of natural  
377 communities to the filtering effects of abiotic conditions and ecological interactions is a  
378 burgeoning field of study worldwide (Mouillot *et al.*, 2013; Ruhi *et al.*, 2018). It has  
379 been especially fruitful in an applied context, in order to understand the response of

380 communities to anthropogenic impacts such as climate change, habitat loss and land use  
381 (e.g. Frainer & McKie, 2015; for stream macroinvertebrates). It has been also used to  
382 disentangle the simultaneous effects of natural and anthropogenic disturbances in multi-  
383 stressed rivers (e.g. Bruno *et al.*, 2016a,b).

384 This study disentangles the separate effects of anthropogenic flow regime alteration and  
385 natural flow intermittence, as well as their interaction, on macroinvertebrate community  
386 functional characteristics in Mediterranean rivers. In general, flow regulation entails a  
387 significant reduction in the mean annual discharges and intensified drought conditions  
388 (Belmar *et al.*, 2013b), which produced some functional patterns common to natural  
389 flow intermittence. Firstly, loss of taxa with semivoltine or univoltine cycles and more  
390 sensitive aquatic stages (i.e., eggs), and the replacement by taxa with multivoltine cycles  
391 and more resistant aquatic stages (i.e., adults). Secondly, reduced functional  
392 redundancy, which could jeopardise the ability of aquatic communities to face an  
393 intensification of flow intermittence caused by climate change or new anthropogenic  
394 disturbances.

395 We found that natural flow regime influenced the functional sensitivity of communities  
396 to human-driven flow alteration, resulting in antagonistic, synergistic and opposing  
397 interaction effects. The effects of flow regulation were more intense in perennial  
398 streams than in intermittent ones given the relevance of antagonistic effects found  
399 between both stressors (considering both trait composition and functional indices). Flow  
400 intermittence imposed an eco-evolutionary pressure on aquatic biota by previously  
401 selecting those resistant or recovery traits (Bonada & Resh, 2013) that conferred  
402 resilience to anthropogenic flow regime alterations.

403    4.1 Responses of functional traits to flow regulation and natural flow intermittence

404    Most of our predictions about the shifts in life cycle-related functional traits caused by  
405    flow regime alteration were confirmed, and common to those produced by flow  
406    intermittence: an increase in taxa with multivoltine life cycles and a decrease in the  
407    univoltine or semivoltine ones. Multivoltine cycles allow life cycle completion in wet  
408    phases by conferring the capacity to recover from floods (Hershkovitz & Gasith, 2013),  
409    after rewetting temporary streams (Bonada *et al.*, 2007b) and, in our study, also after  
410    dam discharges. Thus, the presence of taxa with short life cycles in intermittent streams  
411    confers tolerance to drought intensification as a result of flow regulation, while flow  
412    regulation either eliminates or reduces taxa with semivoltine or univoltine cycles in  
413    perennial rivers (antagonistic effects on life cycle duration).

414    The increased intensity of both stressors involved a reduction in the taxa with aquatic  
415    eggs (the most sensitive stage to floods and dry phases), whose interaction led to an  
416    increase in the taxa with aquatic adult stages (synergistic effect), which commonly  
417    promotes resilience to flow intermittence (see the review by Stubbington *et al.*, 2017).

418    Adult aquatic stages are more tolerant to aquatic habitat fragmentation as the presence  
419    of shrinking pools with harsh environmental conditions (e.g. higher temperatures,  
420    mineralisation and distance among pools) are less suitable for earlier life stages  
421    (Stubbington *et al.*, 2017).

422    Although trait characteristics, such as small size and crawling, promote movement to  
423    the hyporheic zone during drying in intermittent rivers (Stubbington *et al.*, 2017),  
424    reductions in small-sized taxa (0.25-0.5 cm) and crawling locomotion were observed  
425    with increased flow alteration and/or flow intermittence. This could be due to different  
426    causes in each river type. In perennial rivers, the loss of hyporheic habitat caused by  
427    frequent and sudden discharges from dams produced sediment drag and channel

428 incision (Belmar *et al.*, 2013b). In the intermittent streams, water diversion entails dry  
429 channels during long periods below dams and riverbed “terrestrialisation”, with loss of  
430 interstitial refuges (Belmar *et al.*, 2013b), which negatively affects both interstitial taxa  
431 and tegument breathers. In fact, the negative synergistic effect on interstitial taxa found  
432 between both stressors was not surprising in our study area, since interstitial taxa are  
433 typical of mountain streams with little anthropogenic disturbance, perennial flow and  
434 well oxygenated waters (Mellado-Díaz *et al.*, 2008) as well as of intermittent alluvial  
435 rivers (Vander Vorste *et al.*, 2016).

436 Natural flow intermittence had some individual effects different from flow regime  
437 alteration, such as the increase in taxa with diapause or dormancy resistance forms that  
438 promote survival in dry phases (e.g. Gerridae), taxa with clutches in vegetation to avoid  
439 dry phases (e.g. some Odonata), full water swimmers that inhabit receding pools and  
440 predators (e.g. numerous Dytiscidae). The increase in taxa with diapause matched the  
441 expected proliferation of more resistant taxa (Lytle & Poff, 2004; Williams, 2006).  
442 However, Filipe *et al.*, (2013) stated that organisms with scraper feeding habits,  
443 desiccation-resistant eggs and stream drift are common in intermittent streams in  
444 Mediterranean regions, and no significant increase was observed in these trait  
445 characters. At the same time, flow regime alteration also presented some individual  
446 effects not caused by natural intermittence, such as the reduction of aquatic active  
447 dispersion and the decrease of crawlers. The differences in the individual effects  
448 observed between both stressors may be influenced by the different predictability,  
449 duration and spatial patterns of the drying imposed by anthropogenic and natural  
450 hydrologic stress, given the importance of such patterns in intermittent and perennial  
451 streams (Bogan *et al.*, 2015; Aspin *et al.*, 2018; Sánchez-Montoya *et al.*, 2018).

452 The interaction of natural (flow intermittence) and anthropogenic (flow alteration)  
453 stressors may produce opposing effects, like those in shredders, which decreased in  
454 regulated perennial rivers but increased in strictly temporary streams with flow  
455 alteration. This may be caused by natural differences in riparian areas (i.e., leaf litter  
456 quantity and quality) between perennial and intermittent streams that change as a result  
457 of the habitat alteration that flow regulation implies (Belmar *et al.*, 2013b). Riparian  
458 forests are a more important food source in perennial streams (in the absence of  
459 alteration) than in semi-arid intermittent streams, where allochthonous organic matter  
460 input is more limited (Velasco *et al.*, 2003; Hagen *et al.*, 2010). However, when the  
461 “terrestrialisation” associated with anthropogenic flow regulation in intermittent rivers  
462 takes place, particulate organic matter could be available for shredders when flow is re-  
463 established.

#### 464 *4.2 Responses of functional indices to flow regulation and natural flow intermittence*

465 As expected, there was a significant decrease in all functional indices (except FEve) in  
466 response to stress, with a stronger effect for flow alteration compared to flow  
467 intermittence. FDis, FD and FR underwent a non-random (i.e., an effect not attributable  
468 to the underlying taxonomic pattern) decrease as flow regime alteration increased, while  
469 only FR declined in response to natural flow intermittence and their interaction.  
470 Anthropogenic disturbances, such as flow regime alteration, lead to reductions in FRic  
471 and FR (Bruno *et al.*, 2016a, Oliveira *et al.*, 2018), which can diminish ecosystem  
472 resilience to future disturbances (Mouillot *et al.*, 2013). On the other hand, intermittent  
473 streams have often been characterised by lower FRic and FR values than perennial  
474 rivers (Sriever & Lytle, 2006; Suárez *et al.*, 2017), a trend that might be especially  
475 marked during dry periods due to changes in species traits (e.g. Bêche *et al.*, 2006).

476 Functional redundancy was the most sensitive index, as Bruno *et al.* (2016a,b) found  
477 when using riparian vegetation. The antagonistic effect found between both stressors in  
478 FR reflected a condition in which the adaptation of the aquatic community to natural  
479 intermittence mitigated the effect of flow regime alteration, which also occurs with a  
480 natural stressor such as water salinity (Suárez *et al.*, 2017). Redundancy in temporary  
481 streams can reflect the prevalence of taxa with traits that promote resistance and  
482 recovery to flow intermittence (Boersma *et al.*, 2014), and could confer tolerance to  
483 natural and anthropogenic stressors.

484 Our study based on traits at genus level provides interesting results about the interaction  
485 effects of both stressors. However, further research on functional traits at specific and  
486 intra-specific levels is still recommended to refine functional trait responses, given the  
487 uncertainty associated with the use of trait databases (i.e. low availability of traits for  
488 analysis and lack of concordance between measured characteristics and published traits;  
489 Orlofske & Baird, 2014). Future research is also needed to analyze the response to  
490 natural and anthropogenic flow intermittence at larger spatial scales (i.e. among  
491 drainage basins, Heino *et al.*, 2013).

## 492 **5. Conclusions**

493 This study highlights the relevance of considering the interactions of anthropogenic  
494 disturbances (flow regulation) with underlying natural stress (flow intermittence) to  
495 disentangle the ecological processes that take place in complex environments like  
496 Mediterranean rivers, but also in other multi-stressed areas where different  
497 environmental stressors interact. Intensification of flow intermittence and flow  
498 regulation resulted in similar functional responses of macroinvertebrate communities,  
499 like those produced by other natural (e.g. water salinity) and anthropogenic (e.g.

500 agricultural land use) stressors typical of Mediterranean streams. Besides, natural flow  
501 intermittence modulated the functional response of communities to anthropogenic flow  
502 alteration. Antagonistic, synergistic and opposing interactions were observed between  
503 both stressors, thus the effect of flow regulation is conditional to the position along the  
504 flow intermittence gradient. In general, the relevance of antagonistic interactions  
505 (considering both trait composition and functional indices) means that the effect of flow  
506 alteration was stronger in perennial than in intermittent streams, where the aquatic  
507 communities are adapted to natural flow intermittence. In addition, flow regulation and  
508 intermittence also act synergistically fostering some functional strategies to deal with  
509 flow cessation and aquatic connectivity disruption such as resistant stages, but also  
510 removing species sensitive to desiccation as interstitial taxa and tegument breathers. The  
511 detection of such interactions between stressors is highly relevant for ecosystem  
512 management and conservation in Mediterranean streams.

513 The functional approach used in this study and the obtained results could be useful for  
514 other climatic areas where flow intermittence is predicted to increase as a result of  
515 ongoing climate change, which will lead to more extreme and frequent drought  
516 phenomena. In this context, further research to anticipate changes in the stressors  
517 studied or the effect of other potential stressors results essential for water management  
518 and biomonitoring. More attention must be paid to the specific river type and the  
519 functional characteristic monitored when assessing the effects of flow alteration. Given  
520 that the ecological effects of human pressures on aquatic communities could be difficult  
521 to detect in naturally stressed ecosystems such as intermittent rivers, this sort of studies  
522 results of special relevance to disentangle the effects of natural and human pressures, as  
523 well as to identify those traits that could be sensitive to each type of stress. This has  
524 direct applications to design biomonitoring programs and establish conservation goals.

525    **Supporting information**

526    Additional Supporting Information may be found in the corresponding file.

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533    **Conflict of interest**

534    The authors declare no conflict of interest.

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833

## Tables

834 **Table 1.** Functional group (FG) description based on distinctive functional effect traits, including the number of taxa that compose each group.

<b>FG Main effect traits shared by the species within each functional group</b>	<b>Taxa</b>
FG1 Big body size, aquatic active dispersion, predators eating living macroinvertebrates	29
FG2 Small body size, aerial active dispersion, piercers eating living invertebrates	11
FG3 Intermediate body size, swimmers, piercers and predators eating living invertebrates	25
FG4 Heterogeneous body size, full water swimmers and crawlers, shredders and scrapers eating living microphytes and microinvertebrates	26
FG5 Heterogeneous body size, crawlers , shredders and scrapers eating a variety of plant sources	26
FG6 Intermediate body size, crawlers and temporarily attached, shredders, deposit and filter feeders eating dead plants, detritus and living microphytes	23

835

836   **Table 2.** Results of the mixed-effect models showing the best-fitting model equation, *p*-values (only  
 837 significant values, in bold), marginal ( $R^2m$ ) and conditional ( $R^2c$ ) goodness of fit for trait characters that  
 838 presented significant interactions. Pairwise interactions are denoted with an asterisk (A: Flow regime  
 839 alteration; I: Flow intermittence; ‘ns’: non-significant). Interaction effect: A, antagonistic; S, synergistic;  
 840 O, opposing.

Trait	Category	Model equation	A	I	A*I	Effect	$R^2m$	$R^2c$
Life cycle	≤ 1 year	y=0.832-0.077A*I	n.s.	n.s.	<b>0.001</b>	A	0.269	0.285
duration	> 1 year	y=0.739-0.077A*I	n.s.	n.s.	<b>0.001</b>	A	0.269	0.285
Aquatic stages	adult	y=0.438+0.038A*I	n.s.	n.s.	<b>0.004</b>	S	0.239	0.239
Respiration	tegument	y=0.540-0.035A*I	n.s.	n.s.	<b>0.047</b>	S	0.191	0.191
Locomotion and substrate relation	interstitial	y=0.195-0.037I-0.018A*I	n.s.	<b>&lt;0.001</b>	<b>0.034</b>	S	0.338	0.338
Feeding habits	shredder	y=0.620+0.053A*I	n.s.	n.s.	<b>0.045</b>	O	0.143	0.143

841

842   **Table 3.** Results of the mixed-effect models showing the best-fitting model equation, *p*-values (only  
 843 significant values, in bold), marginal ( $R^2m$ ) and conditional ( $R^2c$ ) goodness of fit for the different  
 844 functional diversity indices. Pairwise interactions are denoted with an asterisk (A: Flow regime alteration;  
 845 I: Flow intermittence; ‘ns’: non-significant). Interaction effect: A, antagonistic; S, synergistic; O,  
 846 opposing.

847

Response variable	Model equation	A	I	A*I	Effect	$R^2m$	$R^2c$
FRic	y=0.42-0.07A+0.04A*I	<b>&lt;0.001</b>	ns	<b>0.019</b>	A	0.29	0.32
Fdis	y=0.42-0.022A	<b>0.002</b>	ns	ns	ns	0.15	0.15
FEve	ns	ns	ns	ns	ns	ns	ns
FD	y=0.64-0.04A	<b>0.001</b>	ns	ns	ns	0.15	0.16
FR	y=1.34-0.21A-0.1I+0.08A*I	<b>&lt;0.001</b>	<b>0.025</b>	<b>0.038</b>	A	0.35	0.39

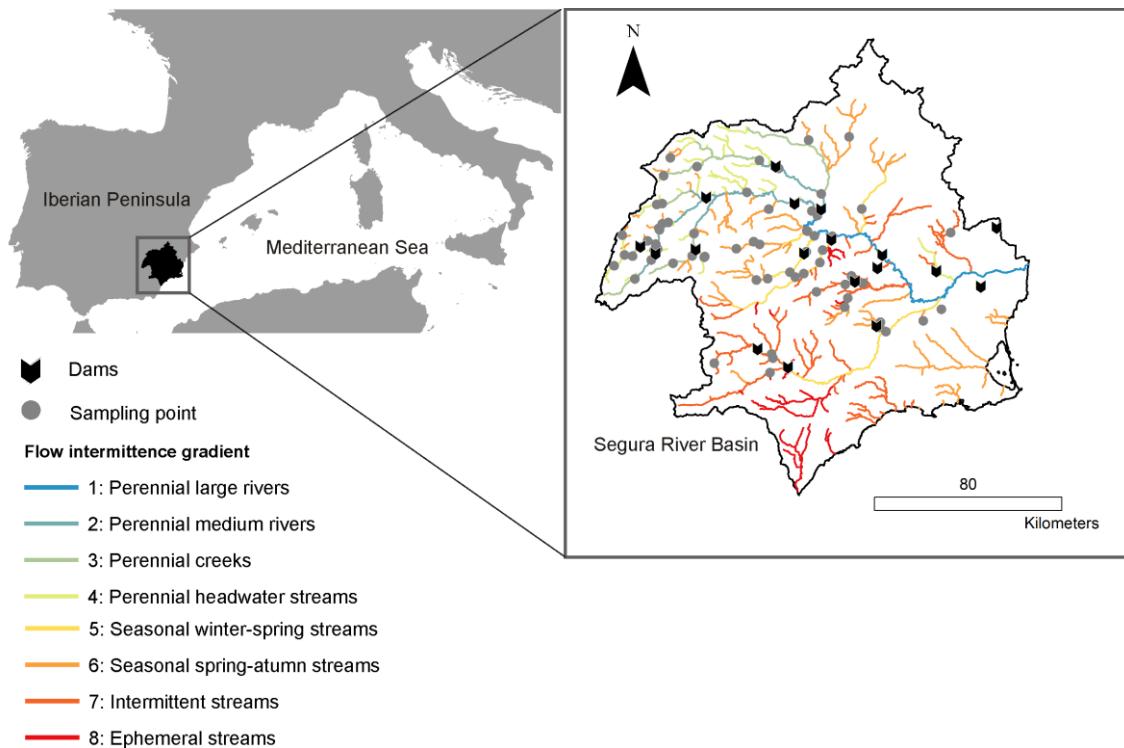
848

849   **Table 4.** Null model results (significant *p*-values in bold) for each tested functional index, selected from  
 850 Table 3 (A: Flow regime alteration. I: Flow intermittence).

Stressor	Functional index			
	FRic	FDis	FD	FR
<b>Z - score</b>				
Intercept	1.44	42.57	128.14	-40.31
A	-0.74	-6.24	-21.86	6.83
I	-	-	-	-4.33
A*I	-0.26	-	-	-4.55
<b>P-value</b>				
Intercept	0.489	<b>0.001</b>	<b>0.001</b>	<b>0.001</b>
A	0.507	<b>0.001</b>	<b>0.001</b>	<b>0.001</b>
I	-	-	-	<b>0.001</b>
A*I	0.889	-	-	<b>0.003</b>

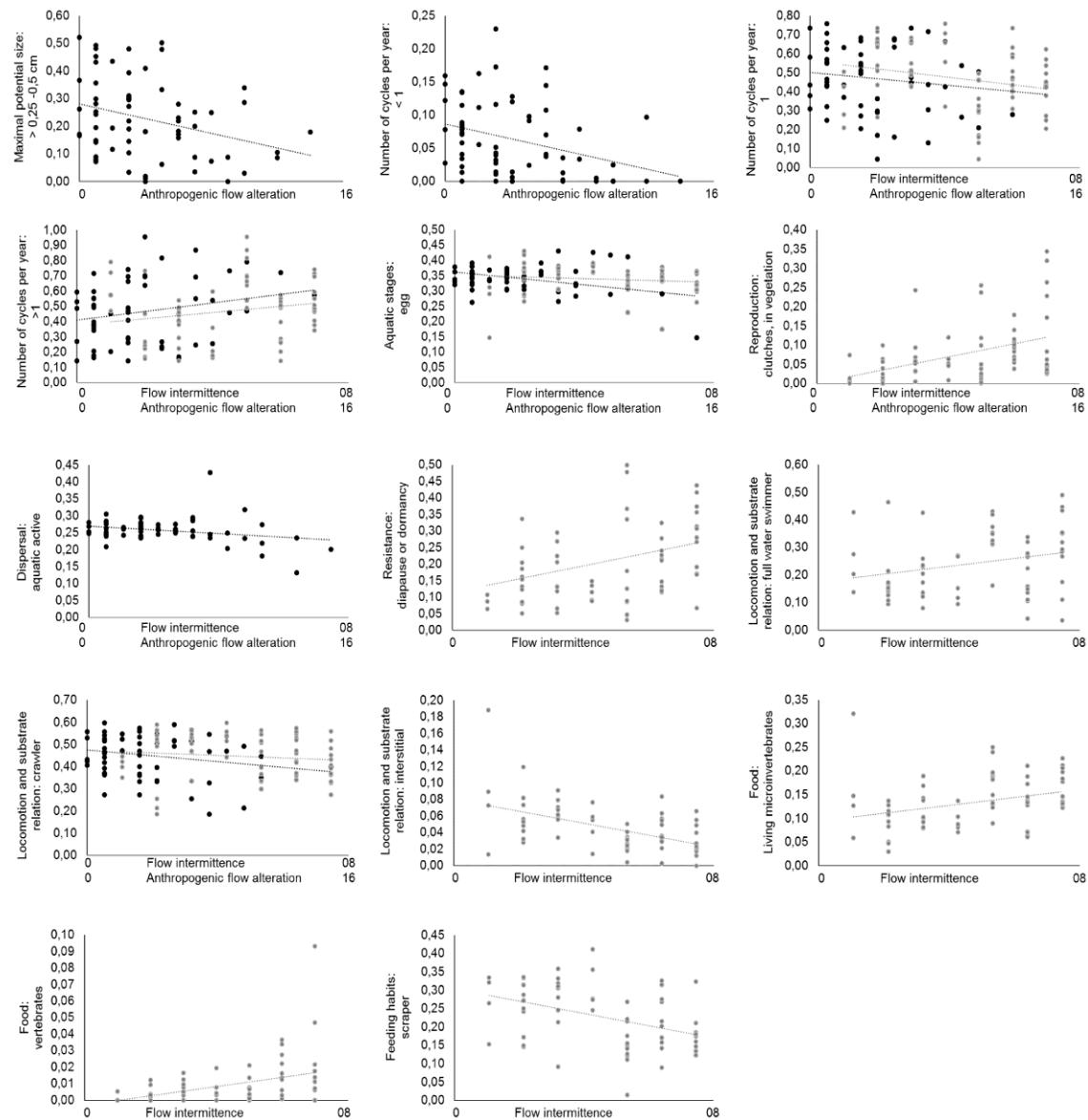
## Figure captions

852 **Figure 1.** Study area showing the sites (grey points) and dams (black arrows)  
 853 on the streams and rivers  
 classified according to the flow intermittence gradient (from “1” or perennial to “8” or ephemeral).



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**Figure 2.** Linear plots between flow intermittence (grey) and anthropogenic flow alteration (black) and proportion of each trait character that obtained statistically significant results for individual stressors. The scale of natural stress has been adjusted to be represented together with anthropogenic stress.



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861      **Figure 3.** Three-dimensional plots between flow intermittence, anthropogenic flow alteration and  
 862      functional characteristics (proportion of each trait character or functional index) that obtained statistically  
 863      significant results for interaction terms.

