Heritability of *Batrachochytrium dendrobatidis* burden and its genetic correlation with development time in a population of Common toad (*Bufo spinosus*)

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We will deposit our data in the Figshare repository for public access.

Abstract/Summary

Despite the important threat that emerging pathogens pose for the conservation of biodiversity as well as human health, very little is known about the adaptive potential of host species to withstand infections. We studied the quantitative genetic architecture responsible for the burden of the fungal pathogen *Batrachochytrium dendrobatidis* in a population of common toads in conjunction with other life-history traits (i.e. body size and development rate) which may be affected by common selective pressures. We found a significant heritable component that is associated with fungal burden, which may allow for local adaptation to this pathogen to proceed. In addition, the high genetic correlation found between fungal burden and development time suggests that both traits have to be taken into account in order to assess the adaptive response of host populations to this emerging pathogen.

Introduction

Emerging diseases have roughly quadrupled over the last 50 years (Jones et al. 2008) threatening biodiversity and human health (Daszak et al. 2000). Pathogens have the potential to induce evolutionary changes in the hosts but the current rates of anthropogenic alteration of the environment may impede this evolutionary response (Longo et al. 2014). Furthermore, while genetic diversity is expected to be high in outbred populations, host organisms may not have enough adaptive potential to develop a rapid evolutionary response to a new selection pressure such as a novel pathogen (Falconer and Mackay 1996). To date, the adaptive potential of wild threatened taxa to respond to pathogen-induced selection is poorly understood.

Previous work estimating the quantitative genetic basis of susceptibility to pathogens has mostly focused either on animal strains for biomedical research (Flint et al. 1995; Rubattu et al. 1996; Råberg et al. 2009) or commercially important species (Price 1985; Roy and Kirchner 2000; Wilfert and Schmid-Hempel 2008). In turn, the complex life-history of many of the studied host species makes it difficult to acquire detailed information about pedigree structure or to undertake informative breeding designs. As a result, most evidence for a genetic basis in susceptibility to pathogens comes from observations of differences in pathogen load among strains/populations and quantitative genetic estimates of broad sense heritabilities (e.g. Mackintosh et al. 2000; McKinney et al. 2011). There is a clear gap in knowledge when it comes to the estimation of the causal quantitative genetic components (i.e. henceforth

genetic architecture, Merilä and Sheldon 1999) for pathogen susceptibility in natural populations. This is in spite of the fact that the additive genetic variance is the ultimate determinants of short-term adaptive responses (Falconer and Mackay 1996; Bürger and Lynch 1997; Mousseau et al. 2000).

Fungal diseases have become a major concern for many taxa in the last decades (Fisher et al. 2012). One of the most important emerging fungal diseases is chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (*Bd*), a pathogenic, virulent, and highly transmissible fungus. At a global scale, *Bd* has infected more than 500 species of amphibians (Aanensen 2007), driving many of them to extinction (e.g. La Marca et al. 2005; Schloegel et al. 2006) and the loss of vertebrate biodiversity associated with chytridiomycosis is the most severe in recorded history owing to a pathogen (Skerratt et al. 2007). Nevertheless, while the spatial epidemiology of the *Bd* panzootic has been the focus of much recent research (Morgan et al. 2007; Farrer et al. 2011; Rosenblum et al. 2013), to our knowledge, no study has yet estimated the quantitative genetic architecture of the fungal load in any amphibian host.

Because traits do not evolve in isolation, it is also necessary to estimate genetic covariances (i.e. the so-called *G* matrix) with other life-history traits that may be under similar selective pressures (Lande 1979; Rose and Charlesworth 1981; McGuigan 2006). In the case of amphibians, body size at metamorphosis and development rate are well studied life-history traits (Berven and Gill 1983; Rowe and Ludwig 1991). They are under the selective influence of environmental factors that may also act on immune response, body condition, and exposure

time to *Bd*, affecting the burden and susceptibility of the amphibians to the pathogen. For instance, environmental conditions both in high altitudes and latitudes have been shown to select for faster embryonic and larval development rates in amphibians (Martin and Miaud 1999; Merilä et al. 2000; Miaud and Merilä 2001; Laurila et al. 2002; Morrison and Hero 2003; Muir et al. 2014). Furthermore, amphibians might present trade-offs between immune system and development time or body size. Costs of accelerating larval development may entail a depression in immune response (Gervasi and Foufopoulos 2008) and the activation of immune defences may engender compromises in the condition of hosts (Garner et al. 2009).

In addition, alpine amphibians are likely to be more affected by chytridiomycosis due to the increased survival and persistence of the fungus and the reduced ability of the host to tolerate the infection in these cold regions (Daszak et al. 1999; Walker et al. 2010). In Central Spain, at the Peñalara Massif (altitude ~2000 m.), *Bd* caused the near-extirpation of the common midwife toad, *Alytes obstetricans*, (Bosch et al. 2001) and mass mortalities in the common toad, *Bufo spinosus* (formerly *Bufo bufo*, (Recuero et al. 2012) (Bosch and Martínez-Solano 2006; Garner et al. 2009; Bosch et al. 2014). Negative population trends have been attributed to chytridiomycosis in a pond of the Peñalara Massif (Bosch et al. 2014). Thus, the Peñalara Massif, within the Guadarrama National Park (GNP), is a unique system that provides the opportunity to monitor the evolution of an amphibian population (i.e. *Bufo spinosus*) from the initial stages of an outbreak of chytridiomycosis.

The aim of our study is to assess whether a wild population has significant adaptive potential to face an emergent pathogen, and determine the phenotypic and genetic correlations with other relevant life-history traits that might influence the evolutionary response to the disease. To that end, we estimated the relative contribution of additive, maternal and dominance effects on the overall phenotypic (co)variation of *Bd*-load, development time and body weight in an amphibian host (*Bufo spinosus*). We strove to estimate the genetic architecture in its most natural setting (in contrast with most quantitative genetic studies of amphibian evolution which estimate heritable parameters in artificial environments (e.g. Berven 1987; Laurila et al. 2002; Uller et al. 2002; Gomez-Mestre et al. 2004), by conducting our experiment under semi-natural conditions *in situ* in ponds that are exposed to natural temperatures and fluctuations in photoperiod.

Material and Methods

Study Area

The study area is a set of three ponds (i.e. Laguna Grande, Laguna Chica, Laguna de Pájaros; detailed location and description in Table I, Appendix 1) of glacial origin located in the Peñalara Massif in Central Spain (from 1800 to 2200 m; Fig. 1). In early spring, *Bufo spinosus* breeds in some of the largest and more permanent ponds in this area and each pond acts as a sub-population.

Sampling and experimental design

140

141 To estimate additive genetic variance and other causal components of
142 phenotypic variation, we collected 21 adults of *Bufo spinosus* in Laguna Grande
143 from July 3rd to 9th 2013, and conducted controlled crosses. We performed 23
144 crosses with 14 males and 7 females in the laboratory. When approximately a
145 quarter of the eggs had been laid by a given female, the male fertilizing the clutch
146 was replaced by a new one. Since the common toad exhibits external
147 fertilization, this protocol assures that the offspring of each female results in half
148 sib families. Each female was crossed with 4 different males, sharing two males
149 with the next female (a scheme of the families that completed the experiment is
150 presented in Table II, Appendix 1). This design allows us to estimate the
151 contribution of each parent, and their interaction, to the phenotypic variance in
152 the offspring. Thus, providing the means to separate dominance, additive and
153 maternal effects in the animal model. This crossed design is the most
154 appropriate in an outbreeding species with no parental care, large clutch size
155 and long pre-reproduction period (sexual maturity at 4-6 years old) (Conner and
156 Hartl 2004). After fertilization each full-sib family was divided in four replicates
157 of ca. 150 eggs.

158

159 To estimate phenotypic divergence among ponds, 2 clutches from Laguna Chica
160 and 7 clutches from Laguna de Pájaros, fertilized in the wild, were collected
161 during the same time period. They were mixed to obtain a pool of eggs for each
162 pond. Every pool was divided in four replicates of ca. 150 eggs.

163

All offspring, both from the quantitative genetic artificial crosses and the clutches collected in the wild, were grown in the laboratory at 15ºC until they reached Gosner stage 25 and then were transferred to Laguna Grande. Fifty randomly selected tadpoles from each replicate were raised in a meshed plastic container of 2L in volume placed randomly into groups of 14 containers (henceforth referred as "block"). A total of 8 blocks formed a raft floating in Laguna Grande almost completely submerged. The raft was secured to the shore with a rope and tadpoles were fed *ad libitum* with ground fish food. A data-logger measured temperature in the pond at ten-minute intervals for duration of the experiment. Temperature differences among containers were slight (maximum difference 0.5ºC, thermometer error ± 0.1ºC). Mesh holes of the containers permitted water exchange but prevented the escape of the tadpoles. This impeded any interaction with other amphibian species. However, transmission of the fungus usually involves infected animals (Piotrowski et al. 2004; Medina et al. 2015) since *Bd* zoospores have low mobility, thus, the containers may significantly reduce exposure to *Bd* spores. Therefore, to mimic natural contact between species, one over-wintered salamander larva from the same pond was introduced into each container for 15 days. As over-wintered *Salamandra salamandra* has been shown to have an infection prevalence of 100% in spring in this pond system (Medina et al. 2015), it was a guaranteed, in this way, to expose experimental animals to infection. Four salamander larvae were sampled after their extraction from the containers and all tested positive for *Bd* as expected (loads ranging from 1.6 and 6.2 GE).

188 After 45 days, we adjusted the density inside the containers to avoid the
189 potential effects of varying densities due to mortality. The tadpoles were
190 collected when they reached Gosner stage 42 (four limbs and tail); they were
191 weighed, photographed, and, finally, euthanized with an overdose of benzocaine
192 and conserved in 96% ethanol.
193
194 *Phenotypic variation*
195
196 To quantify *Bd*-loads, DNA was extracted with the reagent PrepMan Ultra
197 (according to Boyle et al. 2004) from 1063 individual hind limb feet-clips. The
198 extractions were diluted 1/10 before real-time polymerase chain reaction
199 (qPCR) amplification, performed in duplicates, and with *Bd* genomic equivalent
200 (GE) standards of 100, 10, 1 and 0.1, as well as a negative control. The qPCR were
201 performed in a CFX96™ Real-Time PCR Detection System, BIO-RAD. *Bd*-load was
202 measured as the mean of two replicates from the same individual (maximum
203 disparity between replicates= 69,500 GE; mean coefficient of variation per
204 individual = 6,63%; 95% confidence interval = 6.20-7.02%). We considered
205 detection as positive when both duplicates of an individual were positive,
206 amplification curves presented the expected sigmoidal shapes, and the mean was
207 above 0.1 GE.
208 We considered development time as the time inside the pond, thus, from Gosner
209 stage 25 to 42. Some individuals were introduced in the experimental pond
210 within few days of each other due to disparities in reaching Gosner stage 25.
211 Accounting for potential effects of variation in thermal conditions caused by
212 these days, individual development time was measured as accumulated degree-

213 days. We calculated daily temperature averages. Individual development time
214 was the sum of these averages of the days spent inside the pond.
215 For the body mass, each tadpole was weighed (i.e. fresh weight) at Gosner stage
216 42 with a precision balance (±1mg).
217
218 *Neutral genetic variation*
219
220 Toe-clips were obtained for 108 breeding adults from the three study ponds (40
221 from Laguna Grande, 27 from Laguna Chica, and 41 from Laguna de Pájaros)
222 from 2011 to 2013. DNA was extracted with a salt protocol (modified from
223 Aljanabi and Martinez (1997), Appendix 2).
224
225 Microsatellites BbU14, BbU54, BbU13, BbU49, BbU47, BbU24, BbU39, BbU62
226 and BbU23 (Brede et al. 2001) were amplified and divided in three groups to
227 perform three multiplexes following a polymerase chain reaction (PCR) protocol
228 of 2µl of total volume (Kenta et al. 2008) (protocol and PCR conditions in
229 Appendix 2). Microsatellite sequencing was performed using an ABI 3100
230 automatic DNA Sequencer at the DNA Sequencing Unit of the University of
231 Oviedo.
232
233 *Data analysis*
234
235 Distributions of the raw data for *Bd*-load, body weight and development time
236 were slightly log-normally distributed and normality was achieved by a base 10
237 logarithmic transformation. Differences in traits between ponds (i.e. phenotypic

238 divergence) were tested with the ANOVA test implemented in the Stats package
239 of the R software v. 3.0.2 (R Core Team 2013). To test for potential covariation,
240 each pair of traits was fitted sequentially into a linear model in which one of the
241 traits was the response variable and the other was a fixed factor; block was used
242 as a random factor. The linear models were fitted with the lme4 package (Bates
243 et al. 2014).
244
245 To investigate the genetic components of phenotypic variance, we fitted an
246 animal model (Lynch and Walsh 1998) to the data from the offspring of Laguna
247 Grande. This model enabled the separation of genetic (i.e. additive and
248 dominance) from environmental (i.e. common environment/maternal and
249 residual) variance components in *Bd*-load, body weight, and development time.
250 Variances, narrow sense heritabilities, and the proportion of total variance
251 explained by each component were estimated through linear mixed models,
252 using the Bayesian implementation in the R package MCMCglmm (Hadfield
253 2010) in R v. 3.0.2 (R Core Team 2013). Univariate models for each of the three
254 traits were fitted as well as a trivariate model, analyzing all traits simultaneously
255 and providing estimates of the variance components for each trait and the
256 components of covariance between them. Block was used as random factor in all
257 models to account for potential variation caused by the position in the raft. To
258 ensure that the prior distributions did not affect the posterior estimates, we
259 performed a sensitivity analysis. Different priors were tested in all models, i.e.
260 Inverse Wishart priors, conservative priors, flat priors and parameter expanded
261 priors (Supplementary Information). Estimates were obtained from 5,000,000
262 iterations, with a thinning of 100 and a burn in of 1,000,000 iterations.

Convergence was estimated by visual inspection and the Heidelberg and Welch diagnostic. The same convergence criteria were applied for all Bayesian analyses presented in this paper.

The trivariate animal model was specified as follows:

$$\left[\begin{pmatrix} y_{i1} \\ y_{i2} \\ \vdots \\ y_{i1063} \end{pmatrix}, \begin{pmatrix} y_{j1} \\ y_{j2} \\ \vdots \\ y_{j1063} \end{pmatrix}, \begin{pmatrix} y_{k1} \\ y_{k2} \\ \vdots \\ y_{k1063} \end{pmatrix}\right] = X\beta + Z_1\begin{pmatrix} a_1 \\ a_2 \\ \vdots \\ a_{1063} \end{pmatrix} + Z_2\begin{pmatrix} m_1 \\ m_2 \\ \vdots \\ m_{1063} \end{pmatrix} + Z_3\begin{pmatrix} d_1 \\ d_2 \\ \vdots \\ d_{1063} \end{pmatrix} + Z_4\begin{pmatrix} b_1 \\ b_2 \\ \vdots \\ b_{1063} \end{pmatrix} + \begin{pmatrix} e_1 \\ e_2 \\ \vdots \\ e_{1063} \end{pmatrix}$$

Where y_i a column vector containing the phenotypic values for *Bd*-load, y_j a vector containing the phenotypic values for development time and y_k a vector containing the phenotypic values for body weight. The three normally distributed after the normalization by logarithmic transformation. X is a design matrix linking fixed predictors to the data. This matrix has associated vector β, which is the vector of fixed effects, in our case, there were no fixed effects. Z_1, Z_2, Z_3 and Z_4 are the incidence matrices for random effects: additive genetic, maternal, genetic dominance and block effects, respectively. These matrices have associated vectors of coefficients for each animal (individuals from 1 to 1063) representing each random effect contributing to the phenotype. Such vectors of normally distributed random effects are designated as: a for additive genetic, m for maternal effect, d for dominance effect and b is the block effect. The vector e designates residual variation not explained by the factors in the model (Lynch and Walsh 1998). For instance, the model for individual 1 would be:

$$(y_{i1}\, y_{j1}\, y_{k1}) = X\beta + Z_1(a_1) + Z_2(m_1) + Z_3(d_1) + Z_4(b_1) + e_1$$

284 In order to ensure that univariate models could separate additive from
285 dominance effects in the traits, we checked the correlation between the posterior
286 distribution of the additive and the dominance variances.
287
288 For the microsatellite data, scoring errors, large allele dropout and null alleles
289 were checked using Micro-Checker (Van Oosterhout et al. 2004). We used the
290 software Genepop v.4.2 (Raymond and Rousset 1995) to infer the effective
291 number of migrants in the system and its Markov Chain Algorithm (1000
292 dememorisation steps, 1000 batches, 1000 iterations per batch) to test for
293 deviations from Hardy–Weinberg Equilibrium (HWE) and linkage
294 disequilibrium. To estimate genetic drift among sub-populations (i.e. the three
295 ponds) we used two estimators: Weir and Cockerham's F_{ST} as implemented in
296 the software FSTAT v.2.9.3.2 (Goudet 1995), based on expected heterozygosity,
297 and Jost's D_{est}, based on the effective number of alleles (Jost 2008), with SMOGD
298 (Crawford 2010).
299
300 We used microsatellites, pedigree and phenotypic data to study the relative roles
301 of drift and selection on sub-population divergence. Microsatellite data from
302 adults were analyzed with the RAFM package (Karhunen and Ovaskainen 2012)
303 using the R v. 3.0.2 software (R Core Team 2013). A coancestry coefficient
304 matrix for each sub-population was obtained from 1,000,000 iterations, with a
305 thinning of 10 and a burn in of 1,000 iterations. The coancestry matrices were
306 used as the prior for the Driftsel package (Karhunen et al. 2013) also
307 implemented in R v. 3.0.2 software. Driftsel detects whether divergence in trait
308 means deviates from that expected under random drift and could be attributed

to natural selection. Driftsel was run for 1,000,000 iterations, with a thinning of 10 and a burn-in of 1,000 iterations. Experimental block was used as random factor and 1,000,000 tmpmax was fixed for sparse Cholesky decomposition.

Results

Phenotypic divergence

The three traits revealed significant statistical differences among sub-populations (*Bd-load* $p < 0.0001$, $F_{2, 1153.8} = 14.217$; *Development time* $p < 0.01$, $F_{2, 1152.7} = 6.181$; *Body mass* $p < 0.0001$, $F_{2, 1160.3} = 10.035$). The most conspicuous differences among ponds occurred for *Bd*-load, with the offspring from Laguna Chica having a much lower load (Fig. 2; *Bd*-load means and standard errors in GE adjusted for block effects: Laguna Chica – LCH 1292.8 ± 735.38; Laguna de Pájaros - LP 3355.756 ± 806.17; Laguna Grande – LG 2892.94 ± 163.0). All tadpoles tested positive for *Bd*-infection and the loads ranged from 7.6 to 209150 GE in LG, from 44.89 to 10427.5 GE in LCH and from 41.3 to 23865 GE in LP.

Laguna Chica also had the lowest development time (adjusted means and standard errors in accumulated degree-days: LCH 1003.548 ± 27.6249; LG 1087.45 ± 6.123; LP 1045.049 ± 30.28). Albeit statistically significant, the observed differences in development time probably bear little biological relevance.

The offspring from Laguna de Pájaros had the largest mean body mass (adjusted means and standard errors in mg: LP 140.71 ± 4.42; LG 121.21 ± 0.89; LCH 121.91 ± 4.03).

Analysis of phenotypic covariance between traits resulted in a significant positive covariance between *Bd*-load and development time (correlation=0.38; p < 0,XXXX; Fig. 3) but not between any other pairs of traits.

Quantitative genetic architecture

Sequential fitting of the data to animal models with increasing complexity (i.e. additive + residual, additive + dominance + residual, additive + maternal + residual and the complete model estimable: additive + dominance + maternal + residual) showed that the complete model (i.e. additive + dominance + maternal + residual) had the best fit both for the univariate and trivariate cases (i.e. lowest DIC values). All models reached good convergence.

Univariate animal models were insensitive to the prior used for all traits. These models separated additive from dominance effects well in all traits except for body weight. Body weight exhibited a strong negative correlation between the posterior distribution of the additive and the dominance variances, -0.82 (95% confidence interval: -0.8299 – -0.8157). Thus, the estimates of additive and dominance variances for body weight are informative when it comes to the broad-sense heritability but our model could not separate properly the additive from the dominance effects, hence, separate estimates provided in Table 1 have to be taken with caution.

While the posterior distribution of the genetic correlation between *Bd*-load and development time was always accurate and consistent irrespectively of the prior used, the trivariate model yielded flat uninformative posterior distributions for individual trait estimates. This may be due to the difficulty of estimating the joint distribution of components of the G-matrix as compared to the distribution on its individual component separately, given the data available (Ovaskainen et al. 2008; Teplitsky et al. 2014). In this paper, we present the narrow-sense estimates of additive, maternal and dominance variances obtained from the univariate analyses and the genetic correlation from the trivariate model, both using a weak and unbiased prior with an Inverse Wishart distribution (see methods section). Variance estimates (modes) and the proportions of total variance explained are reported in Table 1. All three traits had a strong heritable component. In particular, the mode of the heritability of *Bd*-load (h^2=0.22) for additive effects indicated an important adaptive potential for this trait. Since our tested animals were exposed to *Bd* all the time and, to be sure that the additive estimates of *Bd*-load were not influenced by variation in exposure time we also estimated the heritability of *Bd*-load with development time as covariate. The adjusted heritability still resulted significant (h^2~0.15) and close to the previous value (h^2=0.22). The genetic dominance component of development time (D~0.10) was of similar magnitude as the additive (h^2~0.11). Body weight has a strong broad-sense heritable basis (i.e. additive + dominance) but we could not separate reliably additive from dominance effects. Development time and *Bd*-load presented a significant positive genetic correlation of 0.17 (95% HPDI,

0.0482 - 0.2989). Thus, 0.17 of the overall phenotypic covariation between *Bd*-load and development time in Laguna Grande is due to additive genetic effects.

Genetic drift in the population

No evidence of scoring errors, allelic dropout, or stuttering was detected with Micro-Checker software except for possible null alleles at locus BbU14 in Laguna Grange and Laguna de Pájaros, and loci BbU13 and BbU49 in Laguna de Pájaros. Microsatellite loci conformed with HWE in most cases; only loci with possible null alleles (i.e. heterozygote deficit) had deviation from HWE. We also found slight linkage disequilibrium between BbU13 and BbU47. Analyses with and without these loci resulted in very similar conclusions; therefore, we present the results of the analyses including all microsatellite loci.

Microsatellite data results indicate low divergence between sub-populations, with low F_{ST} = 0.021 (±0.007) and D_{est} = 0.022. Both F_{ST} and D_{est} pairwise values were < 0.04 (table 2). Both estimators found the lowest neutral genetic divergence between the nearest sub-populations: Laguna Chica and Laguna Grande.

Regarding overall migration among ponds inferred by microsatellite data, the effective number of migrants was 6 per generation.

Potential role of natural selection

Both RAFM and Driftsel Bayesian models reached convergence. No significant effect of natural selection on differentiation among sub-population means was

406 revealed by the Driftsel package. All the sub-population trait means were located
407 within the variation range expected under genetic drift. Values of the neutrality
408 test close to 0.5 indicate drift whereas values close to 0 or 1 indicate stabilizing
409 or diversifying selection, respectively. In our case neutrality test resulted in
410 S=0.76, a value not high enough to consider differentiation among sub-
411 populations as caused by diversifying selection.
412
413 **Discussion**
414
415 Our study provides a detailed estimate of the quantitative genetic architecture of
416 infection susceptibility to an emergent pathogen in a natural host population. To
417 our knowledge, this is the first estimate of the adaptive potential to *Bd*-load in an
418 amphibian host. Furthermore, our results revealed a significant genetic
419 correlation between development time of the host and susceptibility to the
420 infection. Further research is needed to better understand the potential joint
421 evolution of these important life-history traits in other populations and taxa.
422
423 *Adaptive potential and genetic correlation*
424
425 We show that a host population can harbor significant evolutionary potential
426 when facing a novel pathogen. Our results suggest that *Bd*-load might respond
427 fast to strong selection since its heritable basis exhibited a predominantly
428 additive component. The maternal effect and genetic dominance for *Bd*-load
429 explained less than 0.01 of the overall phenotypic variation. Development time
430 presented a quantitative genetic architecture with a lower additive component

but larger dominance than *Bd*-load. This indicates that, under similar selection pressure, development time might display a slower evolutionary response (Roff 1997; Lynch and Walsh 1998). However, the response to selection of development time and *Bd*-load should not be considered independently. *Bd*-load and development time showed a significant positive phenotypic covariation in the offspring of Laguna Grande. A portion of this covariation could be due to *Bd*-exposure time, because the slower that development occurs, the greater is the time for keratin to accumulate *Bd* zoospores. In fact, it is well known that the *Bd* panzootic most aggressively affects those amphibian species with longer larval periods and, thus, greater exposure to the fungus (Blaustein et al. 2004; Carey et al. 2006). Our results demonstrated that around 0.17 of the phenotypic covariation is due to genetic correlation. Therefore, the selection on either *Bd*-load or development time may entail an evolutionary response on the other trait. For instance, the strong selection for shorter development time, as could be expected in situations of restricted growth opportunities as mountain environments, high latitudes and, temporal ponds (Miaud and Merilä 2001; Laurila et al. 2002; Muir et al. 2014), might produce genotypes with lower propensity to reach high *Bd*-loads.

Conversely, body weight appears to be able to evolve independently from the other traits since it did not show significant genetic correlations nor phenotypic covariation. Body weight had the largest broad sense heritability and may have a strong evolutionary response to selection. However, our data did not allow for a reliable separation of additive from dominance effects in this trait.

We would like to note that our detailed quantitative genetic estimates come from the population at Laguna Grande and, thus, could be specific to this population. Further population replication, including also lowland and *Bd*-free populations, would be needed to assess the generality of our findings.

Evolutionary processes in the study system

The host population in our system may be dealing with diverse selection pressures. In addition to the new pathogen, common toads are encountering novel environmental conditions in the recently colonized ponds. Laguna de Pájaros and Laguna Chica were occupied by *Bufo spinosus* after the *Bd* outbreak, while Laguna Grande was an established population long before (Bosch and Rincón 2008).

Laguna Grande is the largest pond both in size and with respect to effective population size (28 breeding females captured in 2013 in contrast with 17 in Laguna de Pájaros and 6 in Laguna Chica; J. Bosch unpublished data), therefore, selection pressure being equal in all ponds, a faster rate of adaptation would be expected in Laguna Grande (Soulé 1976; Frankham 1996). Nevertheless, when it comes to phenotypic divergence, Laguna Chica is the most differentiated with the lowest *Bd*-load and development time (Fig. 2). This is the smallest pond, suffering strong thermal fluctuations and risk of desiccation at the end of the summer (J. Bosch unpublished data), which might impose a strong selection towards reducing development time. Due to the genetic correlation between development time and *Bd*-load, low fungal burden could be indirectly selected

481 for. Furthermore, common toads in Laguna Chica might face less infection risk
482 than the other study ponds because of the lack of overwintering salamander
483 larvae, which form the main reservoir of *Bd* in the system after the
484 disappearance of *Alytes obstetricans* (Medina et al. 2015).
485
486 Because adaptive divergence can occur even under moderate gene flow (Endler
487 1973; Rice and Hostert 1993; Garant et al. 2007) we assessed the possibility of
488 adaptive divergence among the toad populations in the three study ponds.
489 Comparison between quantitative genetic and neutral genetic differentiation
490 may help us to assess whether the observed phenotypic divergence is likely to be
491 caused by directional selection (Merilä and Crnokrak 2001; McKay and Latta
492 2002; Leinonen et al. 2008). In our study, the comprehensive tests implemented
493 in Karhunen *et al*. (2013), combining multivariate coancestry and quantitative
494 genetic coefficients, did not provide support for a role of divergent selection in
495 the observed differences. This is not to say that selection is not acting on the
496 studied traits but rather that we cannot discard genetic drift as a relevant cause
497 of the current phenotypic differentiation.
498
499 *Lack of covariation of body size with development or* Bd-*load*
500
501 Body size in ectotherms is expected to have a positive phenotypic relationship
502 with development time (e.g. Laurila et al. 2002; Morey and Reznick 2004; Rudolf
503 and Rödel 2007). However, occasionally, this relationship can also be negative or
504 nonexistent (Travis 1981; Pfennig et al. 1991) which has been related to
505 variation in food availability and mortality rates (Alford and Harris 1988; Rudolf

506 and Rödel 2007). In our study, we did not find any significant phenotypic
507 covariation between the two traits although food availability was not
508 constrained. Due to an unusual heat peak, a high mortality episode was recorded
509 soon after the transfer of the experimental animals to the pond. Dead larvae in
510 the pond decomposed, or were ingested, too fast to measure body size. After this
511 mortality episode, unrelated to *Bd*, no further significant mortality was detected
512 until the termination of the experiment.
513
514 We also explored potential relationships between body weight and *Bd*-load.
515 Previous studies found a negative effect of *Bd*-load (Voyles et al. 2012) and *Bd*-
516 exposure (Garner et al. 2009) on body size in amphibian adults and juveniles
517 respectively. The lack of covariation of *Bd*-load with body weight in our results
518 may imply that negative effects of high *Bd*-loads could become apparent later on
519 in development, although differences in experimental design among studies
520 could also play a role. For instance, since all our tadpoles were exposed to *Bd*, we
521 cannot assess any potential effects of exposure *per se* on body weight. Further
522 studies, linking directly *Bd*-load with mortality, are needed to better understand
523 the defense mechanisms against the pathogen (e.g. resistance and/or tolerance)
524 and its evolutionary relevance.
525
526 *Conclusions*
527
528 Based on the significant genetic correlation between *Bd*-load and development
529 time found in this study, further work in other populations and taxa is required
530 to better disentangle the evolutionary significance of the relationship between

development rates and susceptibility to diseases. This is especially so for taxa with complex life cycles which may present a trade-off between immunity and development rate in time-constrained situations (Rolff et al. 2004) as suggested for amphibians (Gervasi and Foufopoulos 2008).

The heritable component associated with *Bd*-load may allow common toad populations to adapt to chytriodiomycosis. In this context it is necessary to study the strength of *Bd*-induced selection (i.e. estimation of selection coefficients) and the relationship between *Bd*-load, host condition, development rate and mortality in nature.

Given the detailed information since the outbreak of the infection and establishment of new common toad sub-populations, this study sets an initial time point for long-term evolutionary monitoring of the system.

The significant genetic component of *Bd*-load, coupled with the current ease of deep sequencing technologies, makes this species a candidate suitable for mapping the molecular basis responsible for the fungal burden.

Ethics statement

Animal welfare, handling and field permits to conduct this research were obtained from the Consejería de Medio Ambiente, Vivienda y Ordenación del Territorio de la Comunidad de Madrid (permit number: 10/071126.9/13).

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563

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569

570

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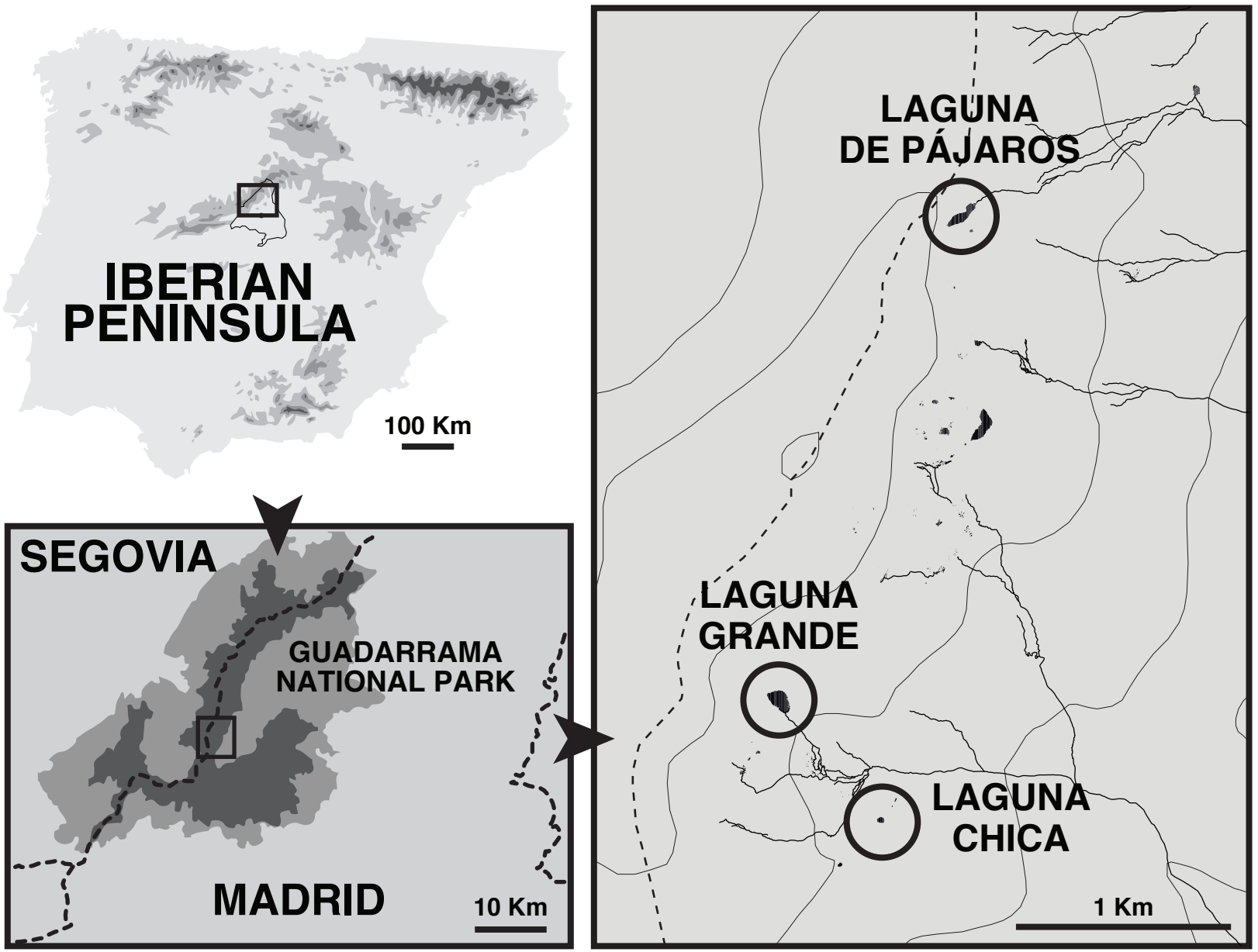
Figure 1: Map of the study area, circles indicate studied ponds.

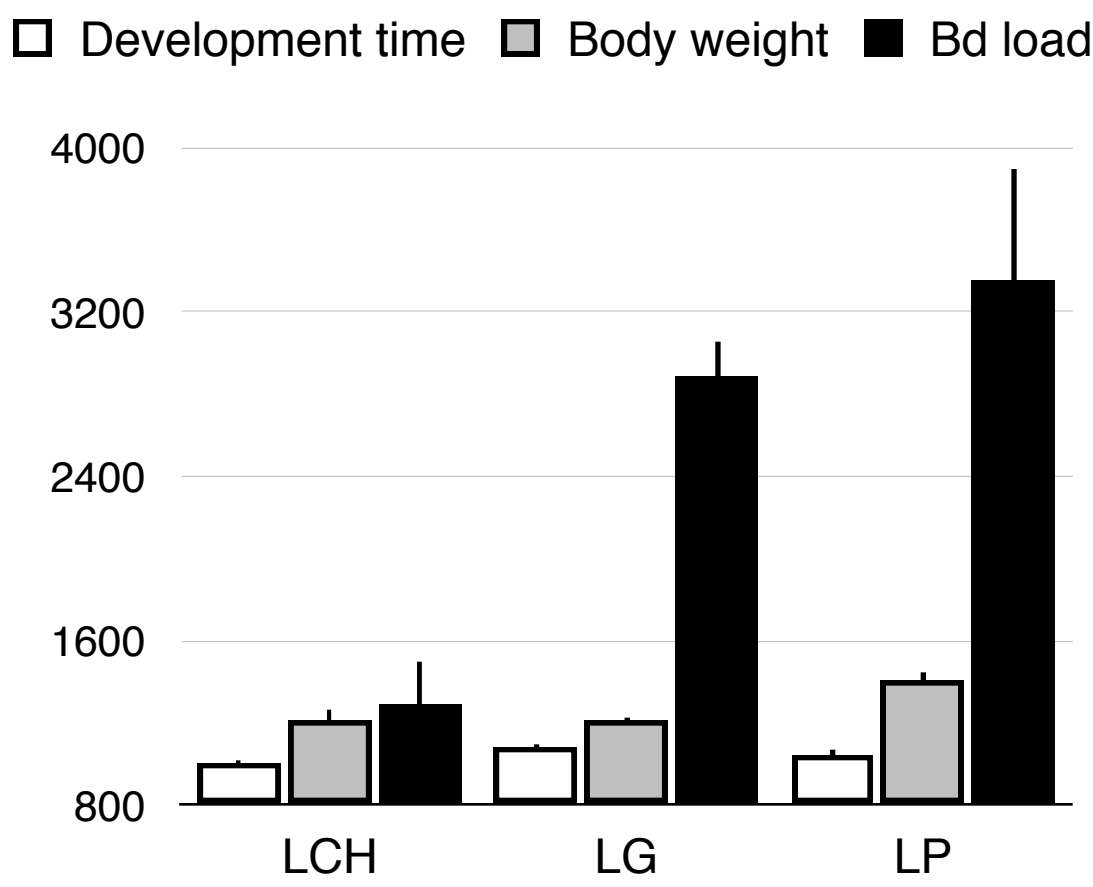
Figure 2. Least square means and SE for accumulated degrees from Gosner stage 25 to 42 (development time), *Bd* genomic equivalents (*Bd*-load), and mg multiplied by 10 (body weight). Pond codes are: LCH, Laguna Chica; LG; Laguna Grande; LP, Laguna de Pájaros.

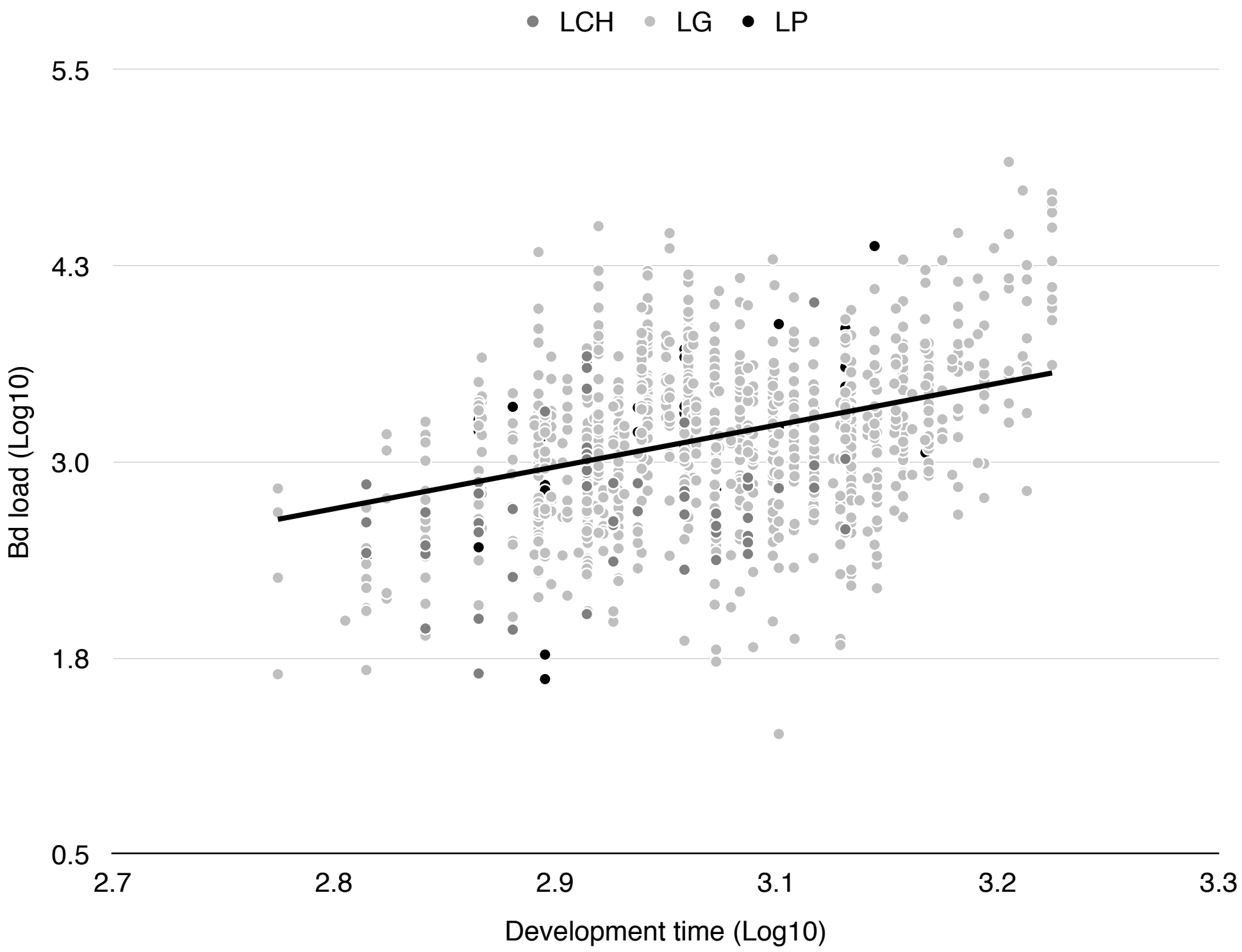
Figure 3. Correlation between *Bd*-load and development time. Each point corresponds to one individual at stage 42. Pond codes are: LCH, Laguna Chica; LG; Laguna Grande; LP, Laguna de Pájaros. The line corresponds to the best linear fit.

Table 1. The upper half of the table shows the mode of the variance explained by the causal components of phenotypic variation followed by their 95% Higher Posterior Density Intervals. V_A, additive variance, V_D, dominance effect, V_M, maternal effect, V_B, block effect, and $V_{R,}$ residual variance. The lower half of the table shows the proportion of the total variance explained by each component, that is the heritability in the case of the additive component.

Table 2. F_{ST} values between sub-population pairs below the diagonal, significant values designed by *. D_{est} values between sub-population pairs above the diagonal. LCH, Laguna Chica; LG; Laguna Grande; LP, Laguna de Pájaros.


IBERIAN
PENINSULA
100 Km
SEGOVIA
GUADARRAMA
NATIONAL PARK
MADRID
10 Km
LAGUNA
DE PÁJAROS
LAGUNA
GRANDE
LAGUNA
CHICA
1 Km


Development time
Body weight
Bd load
4000
3200
2400
1600
800
LCH
LG
LP


LCH
LG
LP
5.5
4.3
3.0
1.8
0.5
Bd load (Log10)
2.7
2.8
2.9
3.1
3.2
3.3
Development time (Log10)

	***Bd*-load**		**Development time**		**Body Weight**	
V_A	0.0546	0.0003 - 0.1610	0.0009	0.0002 - 0.0038	0.0058	0.0019 - 0.0094
V_D	0.0011	0.0001 - 0.0516	0.0010	0.0002 - 0.0036	0.0027	0.0010 - 0.0060
V_M	0.0054	0.0001 - 0.0594	0.0008	0.0001 - 0.0037	-	-
V_B	0.008	0.0016 - 0.0416	0.0004	0.0001 - 0.0017	0.0004	0.0001 - 0.0016
V_R	0.1737	0.1033 - 0.2061	0.0035	0.0013 - 0.0046	0.0007	0.0002 - 0.0021
Additive (heritability)	0.2184	0.0009 - 0.5083	0.1084	0.0273 - 0.4121	0.5523	0.2234 - 0.7922
Dominance	0.0052	0.0005 - 0.1825	0.1042	0.0257 - 0.4295	0.2773	0.0767 - 0.6047
Maternal	0.0048	0.0005 - 0.1879	0.0822	0.0221 - 0.3457	-	-
Block	0.0320	0.0065 - 0.1384	0.0488	0.0160 - 0.1926	0.0336	0.0124 - 0.1394
Residual	0.6528	0.3112 - 0.8142	0.4162	0.1215 - 0.6262	0.0613	0.0158 - 0.2099

F_{ST}	LCH	LG	LP
LCH	-	0.0018	0.0329
LG	0.0053	-	0.0383
LP	0.0325*	0.0232*	-

Appendix 1

Table I. Coordinates, altitude, depth and surface of the study ponds.

	Laguna Grande	Laguna Chica	Laguna de Pájaros
Coordinates	W-3.957451, N40.839877	W-3.951746, N40.834923	W-3.947602, N40.860472
Altitude (m)	2017	1956	2170
Max. Depth (m)	4.7	0.7	1.3
Surface (m^2)	5452	739	4866

Table II: Crossing design. Columns represent females and rows males. Crosses were coded following the code of the parent (i.e. first female and second male). Total number of individuals per family appears below the code.

Female / Male	4	2	8	7	11	6	3
5	Cross 4.5 75 indiv.						
3	Cross 4.3 20 indiv.	Cross 2.3 18 indiv.					
4	Cross 4.4 72 indiv.	Cross 2.4 55 indiv.					
1		Cross 2.1 53 indiv.	Cross 8.1 52 indiv.				
2		Cross 2.2 51 indiv.	Cross 8.2 20 indiv.				
7			Cross 8.7 33 indiv.				
8				Cross 7.8 29 indiv.			
9				Cross 7.9 65 indiv.	Cross 11.9 35 indiv.		
10				Cross 7.10 35 indiv.	Cross 11.10 34 indiv.		
11					Cross 11.11 34 indiv.	Cross 6.11 69 indiv.	
12					Cross 11.12 36 indiv.	Cross 6.12 68 indiv.	
13						Cross 6.13 71 indiv.	Cross 3.13 57 indiv.
14							Cross 3.14 40 indiv.
16							Cross 3.16 41 indiv.

Appendix 2

Lab protocols

Salt extraction protocol

A small piece of toe was incubated overnight at 55ºC with 400µl of salt extraction buffer (TNE 1X and SDS 0.5%) and 8µl of Proteinase K. Next day, 130µl of NaCl 5M were added and the tubes were centrifuged at 10000g for 10min. The supernatant was transferred to another tube and 800µl of 100% cold ethanol was added to compact DNA. Samples were left in the freezer for 2h, centrifuged at 14000rpm for 30 min, and supernatant was discarded. 200µl of 70% cold ethanol was added to wash the DNA and the samples were centrifuged at 14000rpm for 5 min. The pellets of DNA were dried at 55°C for 20 minutes and re-suspended in 100µl of elution buffer (10 mM Tris-Cl and 1 mM EDTA).

Protocol of amplification of microsatellites

To that end, 1µl of DNA was added and dried at the bottom of a reaction tube. Then, 1µl of Multiplex PCR Master Mix (Qiangen) and 1µl of the mix of primers (0.2µM each primer, forward labeled, Apply Biosystem, Life technologies) were added and covered with 5µl of mineral oil. PCRs had the following conditions: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 54 °C (for BbU39, BbU62 and BbU23) or 58°C (for the rest) for 90 s, and 72 °C for 60 s, with a final extension stage of 60 °C for 30 min. PCR products were diluted 1/25.

Supplementary information

MCMCglmm syntax of priors

- Inverse Wishart

prior_univariate= list(G=list(G1=list(V=1, nu = 0.002), G2=list(V=1, nu = 0.002), G3=list(V=1, nu = 0.002), G4=list(V=1, nu = 0.002)),R=list(V=1,nu=0.002))

prior_trivariate= list(G = list(G1 = list(V = diag(3), n = 2.002), G2 = list(V = diag(3), n = 2.002), G3 = list(V = diag(3), n = 2.002), G4 = list(V = diag(3), n = 2.002)), R = list(V = diag(3), n = 2.002))

prior_trivariate2=list(G = list(G1 = list(V = diag(3)*(0.002/2.002), n = 2.002), G2 = list(V = diag(3)*(0.002/2.002), n = 2.002), G3 = list(V = diag(3)*(0.002/2.002), n = 2.002), G4 = list(V = diag(3)*(0.002/2.002), n = 2.002)), R = list(V = diag(3)*(0.002/2.002), n = 2.002))

-Conservative and conditional prior

prior_univ= list(R = list(V = (Variance_trait/5), nu = 0.002), G = list(G1 = list(V= (Variance_trait/5), nu = 0.002), G2 = list(V= (Variance_trait/5), nu = 0.002), G3 = list(V= (Variance_trait/5), nu = 0.002), G4 = list(V= (Variance_trait/5), nu = 0.002),))

prior_triv= list(G = list(G1 = list(V = diag(Variance_traits)/5, n = 2.002), G2 = list(V = diag(Variance_traits)/5, n = 2.002), G3 = list(V = diag(Variance_traits)/5, n =2.002), G4 = list(V = diag(Variance_traits)/5, n =2.002)), R = list(V = diag(Variance_traits)/5, n = 2.002))

-Flat prior

```
prior_univ=list(G=list(G1=list(V=diag(1)*0.01,nu=0.01),
G2=list(V=diag(1)*0.01,nu=0.01), G3=list(V=diag(1)*0.01,nu=0.01),
G4=list(V=diag(1)*0.01,nu=0.01)), R=list(V=diag(1)*0.01,nu=0.01))

prior_triv=list(G=list(G1=list(V=diag(3)*0.01,nu=2.01),
G2=list(V=diag(3)*0.01,nu=2.01), G3=list(V=diag(3)*0.01,nu=2.01),
G4=list(V=diag(3)*0.01,nu=2.01)), R=list(V=diag(3)*0.01,nu=2.01))
```

- Parameter expanded prior (suggested by Jarrod Hadfield)

```
prior_univ=list(G=list(G1=list(V=diag(1),n=1, alpha.mu=rep(0,1),
alpha.V=diag(1)*1000), G2=list(V=diag(1),n=1, alpha.mu=rep(0,1),
alpha.V=diag(1)*1000), G3=list(V=diag(1),n=1, alpha.mu=rep(0,1),
alpha.V=diag(1)*1000), G4=list(V=diag(1),n=1, alpha.mu=rep(0,1),
alpha.V=diag(1)*1000)), R=list(V=diag(1),n=0.002))

prior_triv=list(G=list(G1=list(V=diag(3),n=3, alpha.mu=rep(0,3),
alpha.V=diag(3)*1000), G2=list(V=diag(3),n=3, alpha.mu=rep(0,3),
alpha.V=diag(3)*1000), G3=list(V=diag(3),n=3, alpha.mu=rep(0,3),
alpha.V=diag(3)*1000), G4=list(V=diag(3),n=3, alpha.mu=rep(0,3),
alpha.V=diag(3)*1000)), R=list(V=diag(3),n=2.002))
```