

## ORIGINAL ARTICLE

# Within- and among-population impact of genetic erosion on adult fitness-related traits in the European tree frog *Hyla arborea*

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Assessing in wild populations how fitness is impacted by inbreeding and genetic drift is a major goal for conservation biology. An approach to measure the detrimental effects of inbreeding on fitness is to estimate correlations between molecular variation and phenotypic performances within and among populations. Our study investigated the effect of individual multilocus heterozygosity on body size, body condition and reproductive investment of males (that is, chorus attendance) and females (that is, clutch mass and egg size) in both small fragmented and large non-fragmented populations of European tree frog (*Hyla arborea*). Because adult size and/or condition and reproductive investment are usually related, genetic erosion may have detrimental effects directly on reproductive investment, and also on individual body size and condition that in turn may affect reproductive investment. We confirmed that the reproductive investment was highly size-dependent for both sexes. Larger females invested more in offspring production, and larger males attended the chorus in the pond more often. Our results did not provide evidence for a decline in body size, condition and reproductive effort with decreased multilocus heterozygosity both within and among populations. We showed that the lack of heterozygosity–fitness correlations within populations probably resulted from low inbreeding levels (inferior to ca. 20% full-sib mating rate), even in the small fragmented populations. The detrimental effects of fixation load were either low in adults or hidden by environmental variation among populations. These findings will be useful to design specific management actions to improve population persistence.

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## INTRODUCTION

Understanding the proximal causes leading to population declines and extinctions is a major goal in conservation biology (Frankham *et al.*, 2002; Keller and Waller, 2002). Fragmented populations are threatened to decline mainly because reduction in size and increasing isolation cause demographic stochasticity and genetic erosion (broadly defined as the reduction of genetic diversity due to genetic drift and inbreeding). Inbreeding and genetic drift increase the expression of recessive deleterious mutations and their fixation rate, respectively (Hedrick, 2001). As a consequence, genetically eroded populations are assumed to show a reduced mean fitness. Numerous studies demonstrated in various taxa a reduction in fitness caused by genetic erosion (for example, Rowe and Beebe, 2003; Reed, 2005; Ficetola *et al.*, 2007; Johansson *et al.*, 2007; Angelone, 2010; Luquet *et al.*, 2011b), but they rarely unraveled the genetic process behind this reduction (but see Luquet *et al.*, 2011a). An approach to measure the detrimental effects of genetic erosion on fitness is to estimate correlations between molecular variation and phenotypic performances within and among populations (see Szulkin *et al.*, 2010; Luquet *et al.*, 2011a). Considering both within- and among-population levels is useful to determine the relative importance of genetic processes acting in fragmented populations, and ultimately devise conservation policies (Jaquière *et al.*, 2009; Luquet *et al.*, 2011a).

At the among-population level, correlations between genetic diversity estimators and average fitness traits across a range of populations can reveal ‘ambient inbreeding’ shared by all members of the population (Saccheri *et al.*, 1998; Reed and Frankham, 2003; Luquet *et al.*, 2011a, 2012). The main mechanism behind this effect is the fixation of mildly deleterious alleles by random genetic drift (that is, fixation load; Whitlock *et al.*, 2000; Glemin *et al.*, 2003). It is often this fixation load that poses a serious threat to the persistence of populations because more and more mutations become fixed as population fragmentation increases (Glemin *et al.*, 2003; Whitlock, 2003).

At the within-population level, individual multilocus heterozygosity can be correlated to fitness traits (heterozygosity–fitness correlations or HFCs; David, 1998; Hansson and Westerberg, 2002; Coltman and Slate, 2003; Chapman *et al.*, 2009; Szulkin *et al.*, 2010). Theory predicts that HFC arises if there are differences in pedigree inbreeding (that is, the degree of relatedness between parents) among individuals within populations (Ohta, 1971; Ohta and Cockerham, 1974; Bierne *et al.*, 2000; Tsitrone *et al.*, 2001). HFC should be stronger within populations exhibiting high variance in individual pedigree inbreeding and phenotypic performances, such as recently fragmented populations (Grueber *et al.*, 2008). If genetic erosion is prolonged and extreme, however, HFC becomes harder to detect as genetic variance is eroded and deleterious alleles go to fixation.

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Body size, body condition and reproductive investment are good candidates for revealing genetic erosion effects on the adult life stage since they are closely linked to fitness and likely to involve many different loci that carry deleterious recessive mutations. Body size reflects age (especially in organisms with indeterminate growth) as well as the past growth trajectory of the individual (David *et al.*, 1995), while body condition (usually represented by a function of body mass standardized by body size) represents the nutritional status of organisms, depending more on the recent environmental conditions. Considering both body size and body condition is important to determine if genetic erosion affects growth trajectory or individual abilities to get a good nutritional status. The reproductive investment represents energy allocated to reproduction, usually through mate attraction (secondary sexual traits) or male–male competition in males and offspring production in females. This investment depends on individual size and body condition (for example, Kotiaho *et al.*, 2001; Brandt and Greenfield, 2004; Rasanen *et al.*, 2008). Reduced genetic quality due to genetic erosion may therefore have direct detrimental effects on reproductive investment as well as indirect effects through body size and condition.

As with many amphibians in Europe, European tree frog (*Hyla arborea*) populations have experienced a substantial reduction in size and an increasing isolation during the past decades, and several studies have already revealed genetic erosion in small and isolated populations (Andersen *et al.*, 2004; Angelone and Holderegger, 2009; Broquet *et al.*, 2010). In this context, we investigated the relationship between multilocus heterozygosity at 15 microsatellite loci and body size, body condition and reproductive investment of tree frog adults within and among four wild populations: two were small isolated populations and two others were larger and non-fragmented. Luquet *et al.* (2011a) previously revealed during the early larval period (i) consistent differences in heterozygosity and larval fitness-related traits in these same four populations reared in a laboratory setting and (ii) an increase in larval performance in heterozygous progenies from crosses between two small isolated populations. The resulting positive relationships between genetic diversity and fitness traits among populations provided a sound groundwork to investigate such correlations in the wild on the adult stage in these populations.

We specifically addressed the following question: are body size, body condition and reproductive investment (measured as chorus attendance for males and clutch mass and egg size for females) impacted by genetic erosion within and among populations? As Luquet *et al.* (2011a) observed an effect of the fixation load during the larval period but did not detect inbreeding within the two small

isolated populations, we expected to observe nonsignificant HFCs within populations (that is, no inbreeding depression), including fragmented ones and positive relationships between genetic diversity and adult fitness traits among the four populations (that is, fixation load effect).

## MATERIALS AND METHODS

### Studied populations

Source *H. arborea* populations are located in southeastern France (Table 1; see Luquet *et al.* (2011b) for more details on the studied populations). Two populations are relatively large, located in a non-fragmented landscape, and previously shown to be relatively more genetically variable (Planches P and Mépieu M geographically distant by 8 km; Table 1), while two others are relatively smaller, located in a fragmented landscape, isolated and genetically eroded (Cheylas C and Laissaud L geographically distant by 10 km; Table 1). As shown by Broquet *et al.* (2010), low habitat connectivity for isolated populations acts as a genetic bottleneck by decreasing effective population size.

### Genetic analyses

The individual genetic variability was estimated using buccal swabs performed on each adult sampled in four populations (method according to Pidancier *et al.* (2003) and Broquet *et al.* (2007)). Allelic frequencies were assessed at 15 autosomal microsatellite loci (Ha-A11, Ha-A119, Ha-A127, Ha-A130, Ha-B5R3, Ha-D3R3, Ha-D115, WHA1-20, WHA1-25, WHA1-67, WHA1-103, Ha-D-104, Ha-D-106 and Ha-H-116, Ha-A-136; Arens *et al.*, 2000; Berset-Brändli *et al.*, 2008a,b). Several measures of heterozygosity exist but one or the other are often used without theoretical justification. Szulkin *et al.* (2010) demonstrated that multilocus heterozygosity (MLH) (a simple count of the heterozygous loci) is the most robust measure and does not require standardizations (except to account for a strong variation in genetic diversity among loci; see also Chapman *et al.* (2009) for discussion). We therefore used MLH as the measure of individual heterozygosity.

### Adult sampling and fitness measures

Males and females from each population were caught at night from 16 April to 12 May 2008 when they converged to breeding areas. In the large connected populations, 34/46 (females/males) and 48/36 individuals were collected in P and M, respectively (Supplementary Table S1). In both small isolated populations, we maximized sampling effort to obtain 18/57 (females/males) and 50/157 in L and C, respectively (Supplementary Table S1). All individuals were marked with transponders (Nonatec SA) injected subcutaneously in the back before being released to avoid sampling the same individual.

*Adult body size and body condition.* To estimate body size, we measured the length of the back leg (length between the two metatarsi; precision 0.5 mm) of all sampled individuals. Leg length is a more repeatable measurement on adults than the snout–vent length (JP Léna, personal communication) and is often

**Table 1** Isolation status, coordinates and genetic properties of the study populations

Isolation status	Population	Coordinates	N females	N alleles	Allelic richness	$H_{exp}$	$H_{obs}$	$F_{is}$	$F_{st}$			
									C	L	P	M
Isolated	C	45°23'46"N, 5°59'41"E	30–50	4.00 (0.68)	3.63 (0.62)	0.38 (0.07)	0.42 (0.01)	–0.084	—	0.0978	0.0982	0.0949
Isolated	L	45°28'18"N, 6°3'51"E	15–25	3.60 (0.52)	3.56 (0.51)	0.37 (0.07)	0.38 (0.01)	–0.041	—	0.1177	0.115	
Non-fragmented	P	45°44'20"N, 5°21'7"E	∞	5.60 (0.49)	5.52 (0.47)	0.43 (0.06)	0.40 (0.01)	0.061	—	—	0.0197	
Non-fragmented	M	45°44'3"N, 5°27'21"E	∞	4.73 (0.50)	4.71 (0.50)	0.46 (0.06)	0.46 (0.01)	–0.002	—	—	—	
All isolated				5.13 (0.69)	4.78 (0.65)	0.39 (0.07)	0.41 (0.01)	–0.024				
All non-fragmented				6.07 (0.57)	6.03 (0.57)	0.45 (0.06)	0.43 (0.01)	0.038				

Abbreviations: N females, estimated number of females; N alleles, number of alleles;  $H_{exp}$ , gene diversity;  $H_{obs}$ , observed heterozygosity. Fixation index  $F_{is}$  were all nonsignificant and  $F_{st}$  were all significant. The following genetic information were calculated with  $F_{stat}$  2.9.3 (Goudet, 2001). For more details on genetic characteristics of these populations, see Broquet *et al.* (2010).

used to estimate body size in amphibians (for example, Alho *et al.*, 2011). Body condition was estimated as the residual of body mass (measured after egg laying for females; precision 0.1 g) against leg length with a log–log regression.

**Male reproductive investment.** We estimated the male reproductive investment only in the isolated L population. The complex terrestrial habitat of the C isolated population and the large number of males in P- and M-non-fragmented populations did not allow to capture all males in one night in these populations. We used chorus attendance for each male, that is, the number of nights spent calling at the breeding site, to estimate the male reproductive investment. The chorus attendance is a good proxy for reproductive investment in *H. arborea* because males incur a high metabolic cost while calling to attract females (Grafe and Thein, 2001) and increasing chorus attendance significantly improves male mating success (Friedl and Klump, 2005; Jaquiere *et al.*, 2010). Because males were marked with transponders and released the next day, the first night of capture was not considered to measure chorus attendance. All calling males were sampled each night across all the breeding period (26 nights). We broadcasted a chorus playback to avoid that the calling ceased after male capture or to stimulate the chorus. We stopped the capture session at two o'clock in the morning or later when calling had stopped and pairs in amplexus were not seen for an hour.

**Maternal reproductive investment.** Since *H. arborea* females lay one clutch per reproductive season and do not dispense parental care, their investment in offspring production is crucial for fitness. A good proxy for investment in offspring production is clutch mass and egg size (Rasanen *et al.*, 2008; Ficetola and De Bernardi, 2009). In each population, immediately after capture, we formed random pairs of males and females in transparent tanks (17.5 cm × 33 cm × 18.5 cm) filled with pond water and vegetation to obtain a clutch for each female. We used animals only once for reproduction. The number of clutches obtained from each population varied according to the number of females sampled (Supplementary Table S1). Individuals were released 24 h after mating occurred. As some parts of the clutches were used for other experiments (Luquet *et al.*, 2011b), clutch mass was indirectly estimated by the difference of female body mass before and after laying. To estimate the mean egg size per clutch, 20–30 eggs per clutch were sampled within 24 h after laying (Gosner stage <12) and were stored in formaldehyde (Rasanen *et al.*, 2008). Each clutch sample was photographed with a digital camera (Olympus Camedia C-2000 Zoom, resolution 1600 × 1200 pixels, Center Valley, PA, USA) mounted on a trinocular dissection microscope (Olympus SZX-ILLD200, Olympus, Center Valley, PA, USA). The images were analyzed using ImageJ (<http://rsb.info.nih.gov/ij/index.html>) to estimate the egg area. Because egg size and clutch mass were positively correlated ( $r = 0.36$ ,  $P < 0.001$ ), we used clutch mass and the residuals of egg size against clutch mass regression as indicators of maternal reproductive investment.

**Statistical analyses**

**Power of marker panel.** As HFCs emerge from variance in individual inbreeding, it is useful to estimate this variance directly from molecular data. This can be done through the use of  $g_2$ , a parameter that estimates the identity disequilibrium defined as the excess of double heterozygotes at two loci relative to the expectation under random association (that is, covariance in heterozygosity), standardized by average heterozygosity (David *et al.*, 2007). This parameter is central to all models of inbreeding-based HFC as HFC should exist only if  $g_2 > 0$  (Szulkin *et al.*, 2010). We used the RMES software (David *et al.*, 2007) to estimate  $g_2$  and to test using permutations the null hypothesis that  $g_2 = 0$  (alternative hypothesis  $g_2 > 0$ , expected when there is some variance in inbreeding). In addition, we evaluated the power of our set of markers to detect inbreeding. To that end, we modified RMES to simulate data sets with the same characteristics of our original data sets (number of loci, number of individuals, mean heterozygosity per loci) and various degrees of inbreeding, and in each case we checked how many out of 100 simulations yielded a statistically significant  $g_2$  at a specified  $P$ -value threshold. We chose to simulate inbreeding by assuming a given proportion (10, 20 or 30%) of individuals issued from full-sib mating in the population assuming full-sib mating at equilibrium among generations.

**Correlations between molecular variation and phenotypic performances.** We performed statistical analyses on quantitative traits (leg length, body condition, clutch mass and residuals of egg size) with linear models. Chorus attendance was analyzed with a generalized linear model using a Poisson error distribution with log link. In this generalized linear model, overdispersion has been considered by adding an overdispersion parameter.

To test the relative effects of inbreeding and fixation load, we first analyzed the effects of MLH alone on body size, body condition and reproductive investment with all populations pooled (except for males where reproductive investment was only measured in one population). Second, we fitted a model predicting individual phenotypes as a result of the continuous variable MLH and a categorical factor describing the individual origin (C, L, M and P; except for chorus attendance recorded in only one population). Third, we tested the size and/or condition dependence of reproductive efforts by fitting models with both leg length and body condition, either together or separately. The analyses on body size and body condition were performed separately on males and females because of a strong sex effect on this trait (Supplementary Table S2). The analyses on chorus attendance, clutch mass and residuals of egg size accounted for individual leg length as a covariable.

All nonsignificant interactions were excluded in the final models. The analyses were performed using JMP 7.0 (SAS Institute, Cary, NC, USA).

**RESULTS**

**Variance in inbreeding and power of tests**

All four populations had a positive estimate of  $g_2$ , which was marginally significant only in one of them (M (Table 2),  $P = 0.05$ ), the other three being nonsignificant. Our power calculations showed that we had reasonable power (between 0.5 and 0.8) to detect a level of inbreeding that corresponds roughly to 20% full-sib mating in all four populations—this is approximately the level that corresponded to the only significant value obtained in population M. Higher levels of inbreeding would have been detected with a very high probability (0.9 on average).

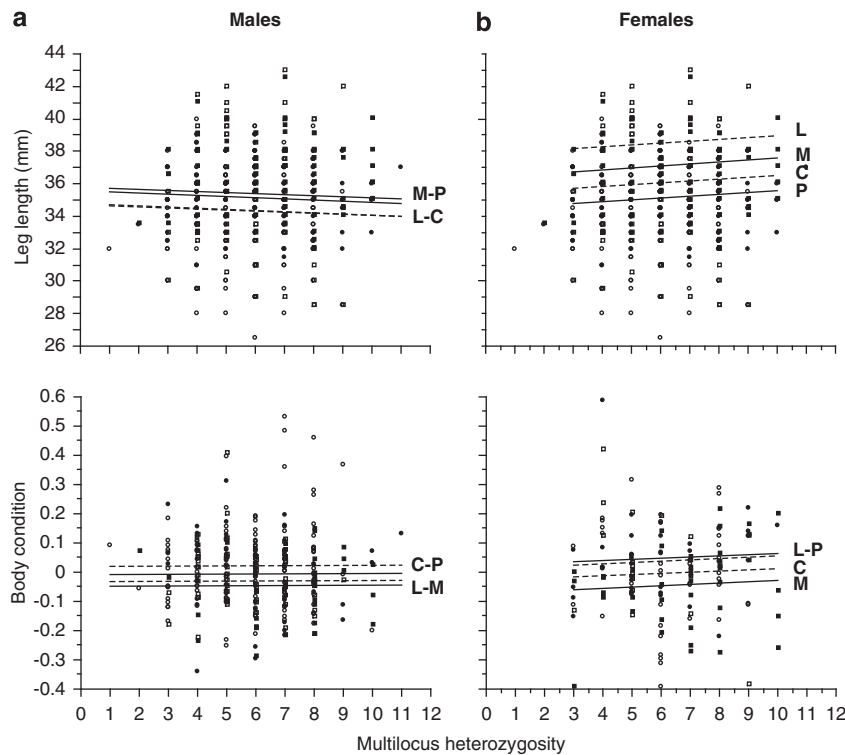
**Correlations between molecular variation and phenotypic performances**

Neither male or female leg length nor condition was associated with MLH, in the whole pooled data set (male leg length:  $F_{1, 293} = 0.099$ ,  $P = 0.754$ ; female leg length:  $F_{1, 147} = 1.665$ ,  $P = 0.199$ ; male condition:  $F_{1, 293} = 0.114$ ,  $P = 0.736$ ; female condition:  $F_{1, 137} = 0.009$ ,  $P = 0.924$ ) and within each population (no significant MLH × population interaction; male leg length:  $F_{3, 287} = 0.298$ ,  $P = 0.826$ ; female leg length:  $F_{3, 141} = 0.212$ ,  $P = 0.888$ ; male condition:  $F_{3, 287} = 1.476$ ,  $P = 0.221$ ; female condition:  $F_{3, 131} = 1.582$ ,  $P = 0.197$ ; Figure 1). Leg length and body condition of males and females significantly varied among populations (Supplementary Table S2 and Supplementary Table S3 for coefficients).

**Table 2** Observed values of  $g_2$  and power of the marker panel to detect various levels of inbreeding

	Populations			
	L	C	P	M
Observed $g_2$ ( $P$ -value)	0.006 (0.31)	0.005 (0.23)	0.008 (0.26)	0.019 (0.05)
<i>Power of test under FSM rate (expected <math>g_2</math>)</i>				
0.1 (0.007)	0.2	0.57	0.16	0.2
0.2 (0.018)	0.52	0.79	0.54	0.64
0.3 (0.032)	0.74	1	0.83	0.92

Abbreviation: FSM, full-sib mating. The power is the probability to have a significant  $g_2$  parameter ( $P < 0.05$ ) for each population assuming recurrent FSM at equilibrium (rates 10, 20 and 30%) from 100 simulations (RMES software; David *et al.*, 2007).



**Figure 1** The relationships between leg length, body condition and multilocus heterozygosity of (a) males and (b) females in the four populations. Dashed and full lines represent, respectively, individuals from isolated (C, open circle; L, open square) and connected (P, filled circle; M, filled square) populations. Points are individuals.

Chorus attendance of males from the L population globally increased with leg length (low for small leg length and highly variable among males with large leg length;  $\chi^2_1 = 7.75$ ,  $P = 0.005$ ; Figure 2a). There was no significant relationship between chorus attendance and male condition ( $\chi^2_1 = 1.47$ ,  $P = 0.225$ ; Figure 2a).

Clutch mass was positively associated with female leg length in the four populations, while a positive association between clutch mass and body condition was found only in the L-fragmented and M-non-fragmented populations (significant female condition  $\times$  population interaction; Figure 2b, Tables 3a and b and Supplementary Table S4 for coefficients). Egg size significantly increased with female leg length and condition in the four populations (Figure 2b and Tables 3a and b). However, unlike leg length, body condition was not a good predictor of female reproductive investment; female condition did not explain a significant amount of variance when leg length was present in the model (Table 3c).

Although chorus attendance was highly variable among males from the L isolated population, from 1 to 10 nights, this male reproductive investment was not correlated with MLH (MLH:  $\chi^2_1 = 0.054$ ,  $P = 0.816$ ; male leg length:  $\chi^2_1 = 7.475$ ,  $P = 0.006$ ; Figure 3a). Similarly, clutch mass and egg size were not affected by MLH in any of the models (Figure 3b and Table 3d). This was true for all populations (nonsignificant MLH  $\times$  population interaction; Table 3e).

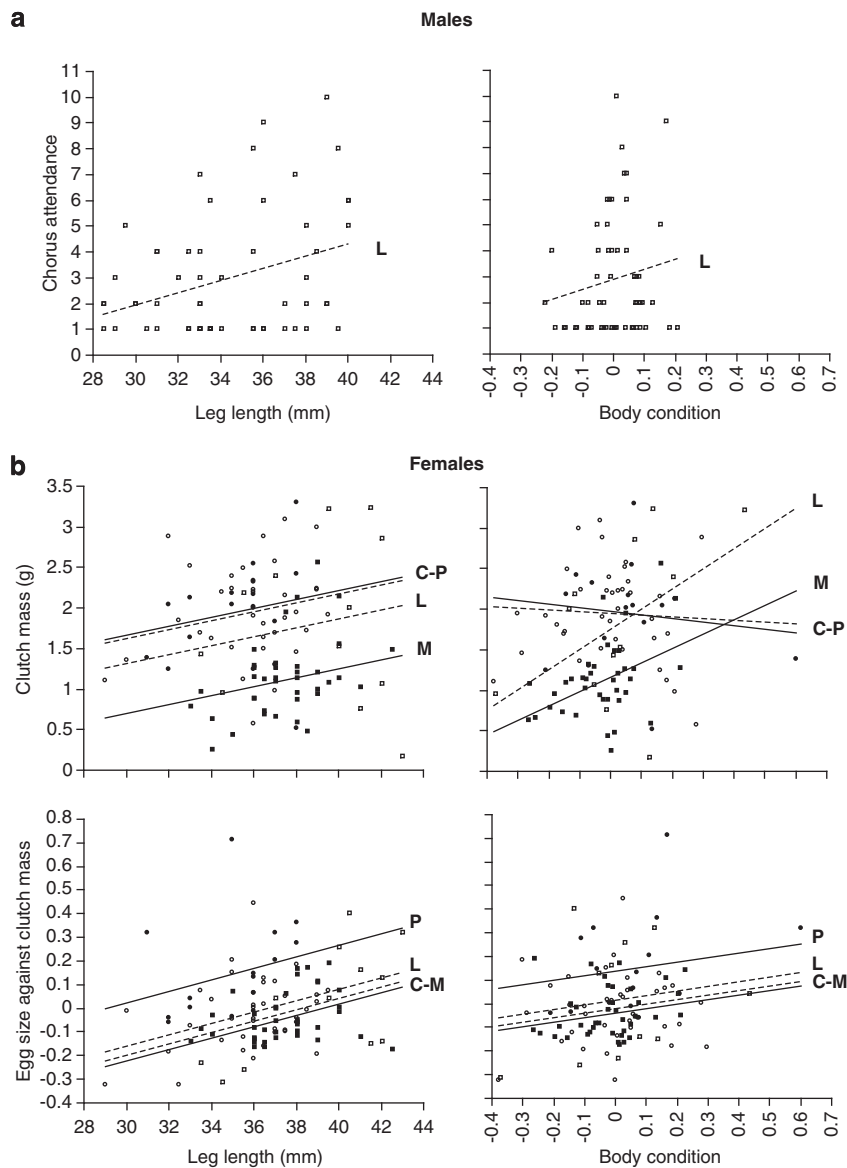
## DISCUSSION

Our study investigated how genetic erosion (that is, reduction in genetic diversity due to inbreeding and genetic drift) in fragmented populations could impact adult size, condition and reproductive investment of males and females. None of these traits (size, condition and reproductive investment) were positively correlated with multilocus heterozygosity, be it within or among populations.

## Lack of HFCs within populations: methodological limitations or low inbreeding levels

The lack of HFCs on adult traits within populations (especially in fragmented ones) can have several non-exclusive origins. First, if variance in inbreeding levels within populations is too low, HFC disappears both because inbreeding explains a smaller proportion of the variance in fitness expressed in the population, and because marker heterozygosity becomes more loosely correlated with inbreeding (Pemberton, 2004). Second, HFC and inbreeding might be present, but the number and genetic diversity of the markers used might be insufficient to capture them adequately. Third, the traits studied may be relatively insensitive to inbreeding. The first and second hypotheses can be evaluated using our estimates of  $g_2$  and associated simulations. The  $g_2$  values in our populations were positive but small, suggesting very moderate levels of inbreeding, equivalent to 10% full-sib mating, except in the M population (20%). Our simulations showed that our set of 15 markers was likely to detect inbreeding levels equivalent to 20% full-sib mating or more (Table 2). It is therefore probable that inbreeding levels were truly low in these populations, even the small ones, which partly explains why HFCs were not detected. Lack of HFC within populations was also found in a previous study on larval traits in the same four populations (Luquet *et al.*, 2011a), using a more powerful estimate of inbreeding (that is, average expected heterozygosity in offspring from parental genotypes; Primmer *et al.*, 2003).

Low frequency of matings between related individuals is a possible cause of low overall impacts of inbreeding depression. Importantly, the European tree frog exhibits complex mechanisms of female mate choice (for example, Jaquier *et al.*, 2010) that could lead to inbreeding avoidance, especially in small fragmented populations (Neff and Pitcher, 2005).



**Figure 2** The relationships between reproductive efforts and leg length or body condition of (a) males (chorus attendance) and (b) females (clutch mass, egg size) in the four populations. Dashed and full lines represent, respectively, individuals from isolated (C, open circle; L, open square) and connected (P, filled circle; M, filled square) populations. Points are individuals.

The low HFC within populations could also be due to the lack or weakness of the segregational load affecting the traits under study. Early-expressed traits such as embryonic development and egg survival are often more affected by segregating deleterious alleles (and inbreeding depression) than adult traits. Mutations that disrupt basic metabolic or developmental processes when in the homozygous state are often semilethals and the animals die before reaching the adult stage; hence, they cannot contribute to HFC. Ficetola *et al.* (2010) demonstrated in a small and isolated population of the threatened frog *Rana latastei* that rapid selection against inbred individuals decreased the inbreeding coefficient ( $F_{IS}$ ) and increased heterozygosity between hatching and metamorphosis. Such selection against unfit genotypes along the life cycle should decrease the phenotypic variance in aging cohorts, which makes the HFC harder to detect (David, 1998; see example in David *et al.* (1997)). The

phenotypic variance ( $CV^2$ ) was indeed low within populations (ca. <5% except for clutch mass; see Supplementary Table S2) that confirms a low level of inbreeding depression in the traits under study (Waller *et al.*, 2008).

#### Genetic diversity–mean fitness trait correlations among populations

Across the range of populations, genetic diversity was not related to average adult fitness traits measured in the wild, which contrasted with the positive relationships detected between average expected heterozygosity in offspring and average larval fitness traits (Luquet *et al.*, 2011a). The effects of fixed deleterious recessive alleles previously revealed during the larval stage were lacking or were not detected in adults. This difference may be explained by three hypotheses.

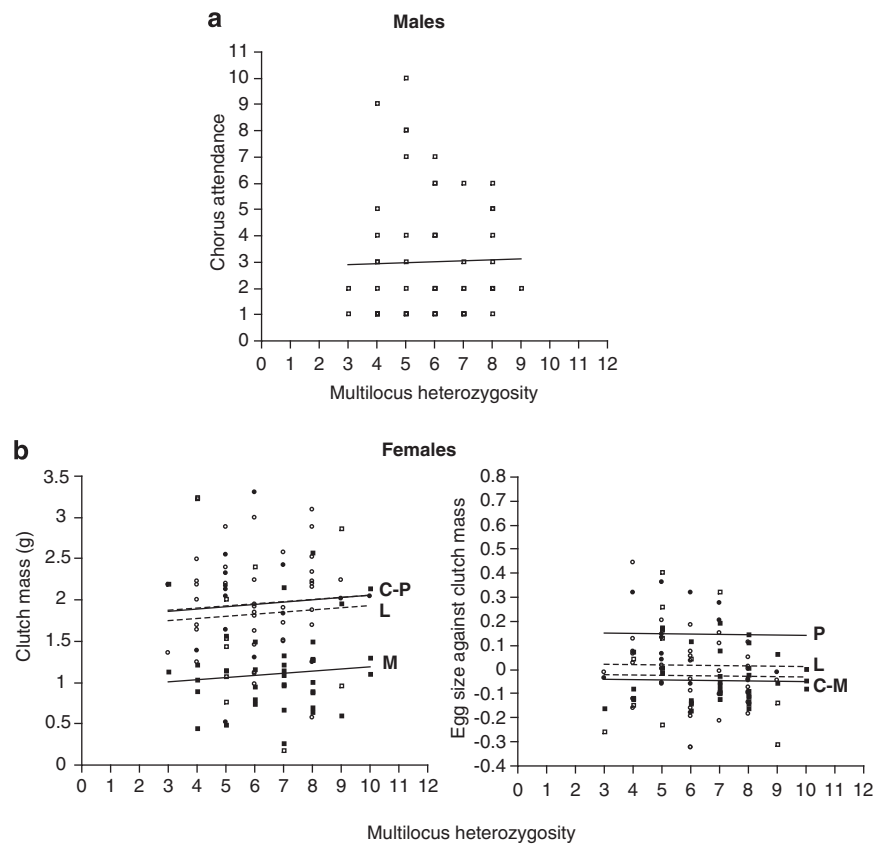
**Table 3** Linear model analyses of variance for clutch mass and egg size (residuals of egg size against clutch mass) on (a) female leg length and population origin, (b) female condition (that is, residuals of body mass against leg length log–log regression) and population origin, (c) female leg length, condition and population origin, (d) MLH and female leg length and (e) MLH, female leg length and population origin

	Clutch mass				Residuals of egg size against clutch mass			
	Num. d.f.	Den. d.f.	F	P-value	Num. d.f.	Den. d.f.	F	P-value
<i>(a)</i>								
Female leg length	1	98	4.515	0.036	1	98	14.275	<0.001
Population	3	98	16.057	<0.001	3	98	9.545	<0.001
<i>(b)</i>								
Female condition	1	95	5.059	0.027	1	98	3.508	0.064
Population	3	95	12.67	<0.001	3	98	4.934	0.003
Female condition × pop.	3	95	3.198	0.027				
<i>(c)</i>								
Female leg length	1	97	4.326	0.040	1	97	14.079	<0.001
Female condition	1	97	2.659	0.106	1	97	3.426	0.067
Population	3	97	14.833	<0.001	3	97	8.246	<0.001
<i>(d)</i>								
Female MLH	1	100	0.220	0.639	1	100	0.877	0.351
Female leg length	1	100	0.581	0.810	1	100	4.817	0.031
<i>(e)</i>								
Female MLH	1	97	0.608	0.437	1	97	0.103	0.749
Female leg length	1	97	4.449	0.037	1	97	9.129	<0.001
Population	3	97	16.088	<0.001	3	97	14.176	<0.001

Abbreviations: d.f., degrees of freedom; MLH, multilocus heterozygosity; pop., population.

Nonsignificant MLH × population interaction has been removed of the final models. Estimates and standard errors are presented in the Appendix (Supplementary Table S4).

Bold values indicate significant effects.



**Figure 3** The relationships between reproductive efforts and multilocus heterozygosity of (a) males (chorus attendance) in the L-fragmented population and (b) females (clutch mass, egg size) in the four populations. Dashed and full lines represent, respectively, individuals from isolated (C, open circle; L, open square) and connected (P, filled circle; M, filled square) populations. Points are individuals.

First, the expression of fixed deleterious alleles can be specific to a life stage or an environment (Ouborg *et al.*, 2010). Especially in species exhibiting complex life cycle like amphibians, the gene pools expressed during larval and adult stages, in aquatic and terrestrial habitats, respectively, can have different fixation loads and therefore different detrimental effects (Husband and Schemske, 1996; Koelewijn *et al.*, 1999; Escobar *et al.*, 2008).

Second, in this study, the adult traits were measured on wild adult individuals varying in age and experiencing different environmental conditions, while in Luquet *et al.* (2011a), the larval traits came from laboratory experiment in controlled conditions. Environmental variations or differences in age–structure among populations may override the genetic effects on phenotypes (Slate and Pemberton, 2006; Szulkin *et al.*, 2010; Luquet *et al.*, 2011a). Our results support this idea. Reproductive investment was highly size-dependent (that is, larger males and females were able to invest more in reproduction), while the effect of body condition was not significant when body size accounted for. This suggests that reproductive investment was more dependent on the age of individuals and their past growth trajectory (which affect body size) than on recent environmental conditions (which affect current nutritional status and body condition). Moreover, the significant differences in average body size, body condition and reproductive investment among populations were not related to the fragmentation status, for example, females of the L-isolated and P-non-fragmented populations had similar body condition and were both larger than those in the remaining two populations (one isolated and one non-fragmented; Supplementary Tables S2 and S3). Ethical considerations prevented us from performing skeletochronology (which requires toe clipping) to estimate age of individuals. Several environmental factors could be responsible to these variations among populations, but determining these factors was beyond the scope of this study. A challenging experiment would be to compare in controlled conditions the detrimental effects of fixation loads at different life stages from embryos to adults on the same cohorts (see Escobar *et al.*, 2008).

## CONCLUSION

Examining how fitness in wild populations is impacted by inbreeding and genetic drift is a major goal for conservation biology. Our study showed how it can be a difficult challenge in the wild when individuals have experienced different environmental conditions. The relationships between multilocus heterozygosity and adult fitness traits that we investigated within and among populations were not significant; however, it is important to publish such negative results in this field because it is likely to suffer from a publication bias. The lack of relationships is as interesting as positive correlations because both can represent a biological reality. Here, we showed that the lack of HFCs within populations probably resulted from low inbreeding levels (inferior to our marker panel resolution, that is, 20% full-sib mating rate), even in the small fragmented populations. The detrimental effects of fixation load were either low in adults or hidden by environmental variation among populations and further work is needed to unravel the relative effects of genetic and environmental variations on adult phenotypic variation. In a conservation perspective, our study, together with our previous study on tadpoles (Luquet *et al.*, 2011a), highlighted that these populations are more likely to lose fitness from the expression of fixation load during the larval stage than from within-population variation in inbreeding. These findings will be useful to design specific management actions to improve population persistence.

## DATA ARCHIVING

Data have been submitted to Dryad repository: doi:10.5061/dryad.p0d83.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (<http://www.nature.com/hdy>)