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Can antioxidant responses be induced by habitat fragmentation process?

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Animal populations are increasingly forced to live in small residual natural or semi-natural areas due to habitat loss and fragmentation. Here, the viability of populations is often compromised by intrinsic threat factors typical of small and isolated populations, such as inbreeding depression, genetic drift and environmental and demographic stochasticity. Under these circumstances, organisms may have low fitness due to inadequate physiological responses needed to face environmental challenges. However, few studies have investigated the relationship between habitat fragmentation and stress defences. In this study, we aimed to test whether an increase in the level of individual inbreeding produced an increase in the antioxidant system response. To this purpose, we genotyped 151 individuals of fire salamander *Salamandra salamandra* (Amphibia: Urodela) within five sampling populations, located in forest landscapes with different degree of fragmentation in northern Italy. For 113 individuals we also measured the glutathione-S-transferase (GST) and catalase (CAT) enzyme activity. Results showed a significant increase in individual GST activity for increasing levels of inbreeding, whereas no relationship was found for CAT. We also measured acetylcholinesterase to test the possible confounding effects of pesticides that might have occurred in fragmented landscapes with forests interspersed with agricultural areas. However, no difference in this enzyme activity was found among sampling populations. We argue that high levels of GST activity may be symptomatic of oxidative stress derived from inbreeding. An increased frequency of homozygous deleterious alleles due to inbreeding may cause homeostatic alterations and trigger the expression of GST for protection against hydrogen peroxide reactive oxygen species. We suggest using GST as a biomarker for environmental stressors with great caution and not to underestimate that the sources of stress deriving from habitat fragmentation could lead to an unbalance in the oxidative status, possibly increasing population susceptibility to infectious diseases and, potentially, spillover events and zoonoses.

Keywords: glutathione-S-transferase, inbreeding, isolation, landscape fragmentation, landscape immunity, *Salamandra salamandra*

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Introduction

Reactive oxygen species (ROS) are by-products of aerobic mitochondrial metabolism (Kurutas 2015). The unbalance between ROS production and catabolism leads to an 'oxidative stress', associated with a potential cytotoxic damage due to oxidation of macromolecules and impairment of their biological function (Sies 1986, Handy and Loscalzo 2012, Halliwell and Gutteridge 2015). ROS overload can be induced by several immune stimuli and by physical (e.g. heath and drought, Vinagre et al. 2012, Schweizer et al. 2019) or chemical (e.g. pesticides and heavy metals; Rainio et al. 2013, Aliko et al. 2018) environmental stressors. Activation of stress responses could also be promoted by genetic stress (Kristensen et al. 2005, Pedersen et al. 2008, Reed et al. 2012, Zhao et al. 2019). Overexpression of Hsp70, a highly conserved heat-induced chaperone, occurs under inbreeding conditions and is negatively associated with fitness traits (Kristensen et al. 2002, Pedersen et al. 2005, Cheng et al. 2006, Okada et al. 2011).

Animal populations inhabiting fragmented landscapes are often divided into small and isolated sub-populations (Hanski and Simberloff 1997). Hostile matrices drastically reduce dispersal movements between habitat patches (Dondina et al. 2018a, 2019) and make populations more vulnerable to inbreeding and genetic drift (Tischendorf and Fahrig 2000, Lowe and Allendorf 2010). The relationship between habitat fragmentation and stress responses in wildlife has not been deeply investigated and previous studies have mainly focused on physiological responses to stress such as the stimulation of glucocorticoid-mediated metabolism (Suorsa et al. 2003, Martínez-Mota et al. 2007, Johnstone et al. 2012, Carlitz et al. 2016).

Amphibians are good model candidates for studying the effects of habitat fragmentation (Moore et al. 2011) because of their low dispersal ability (Schulte et al. 2007, Allentoft and O'Brien 2010) and high philopatry (Blaustein et al. 1994). The fire salamander *Salamandra salamandra* with its strict ecological requirements, for both aquatic and terrestrial phases (Schmidt et al. 2005) and low dispersal ability (Denoël 1996, Schulte et al. 2007) is a putative model species to investigate physiological stresses related to inbreeding depression due to isolation of populations (Bani et al. 2015).

In this work we examined the activity of antioxidative enzymes in fire salamander. Our hypothesis was that antioxidative responses could be triggered by genetic stress derived from inbreeding conditions due to fragmentation-induced isolation. We asked whether an increase in the individual activity of glutathione S-transferase (GST) and catalase (CAT), two enzymes promoting protection from oxidative stress, could be related to the level of inbreeding.

Methods

Study area, sampling design and collection of biological samples

The study area is the Prealpine belt of Lombardy (northern Italy, Fig. 1) where broad-leaved forests are fragmented due to urban sprawl, especially in the foothill area. In a previous work conducted by Pisa et al. (2015), a representative

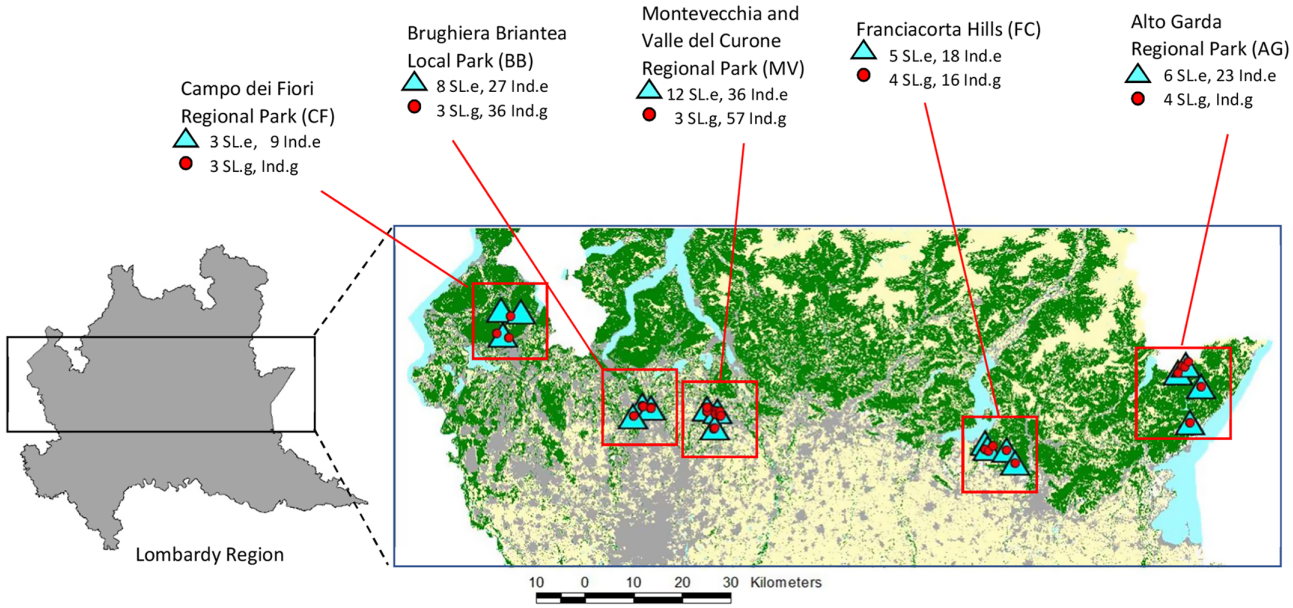

Figure 1. Study area in Lombardy Region (northern Italy). Cyan triangles: enzyme activity sampling locations (SL.e: number of sampling locations, and Ind.e: number of individuals sampled for enzyme activity analysis); red dots: genetic sampling locations (SL.g: number of sampling locations, and Ind.g: number of individuals sampled for genetic analysis); red squares: sampling populations (SPs); background colours: green, forest areas; light yellow, open areas; grey, urban areas; light blue, water bodies. Percentage of forest cover within the area of each sampling population: CF, 85%; BB, 48%; MV, 47%; FC, 81%; AG, 80%.

genetic sample of fire salamander sub-populations (i.e. sampling populations, SPs) was collected in forests with a different degree of fragmentation. In five SPs included in the study by Pisa et al. (2015) (Campo dei Fiori Regional Park [CF], Brughiera Briantea Local Park [BB], Montevecchia and Valle del Curone Regional Park [MV], Franciacorta Hills [FC], Alto Garda Regional Park [AG]; Fig. 1), we conducted another study aimed at assessing biomarker activities.

In order to combine the information from the two studies, we selected three to four sampling locations (SLs, 17 in total), where 16–57 individuals (151 in total) were sampled for genetic analyses, and three to 12 SLs (34 in total) where 9–36 individuals (113 in total) were sampled for enzyme analyses. For details regarding the population genetic analysis (e.g. storage, preservation, extraction and sequencing) see Pisa et al. (2015).

Tissue samples were obtained by cutting off the tip (approximately 3–4 mm) of the larvae's tail and storing them in liquid nitrogen. Biological samples were collected throughout the year, from 2010 to 2013. As the larval stage (new-born or overwintering individuals) could affect the enzyme activities (Beaulieu and Costantini 2014), we noted this information for each individual sampled. To account for the possible effect of water quality on enzyme activities, in each SL, we measured water temperature, O_2 saturation, pH, conductivity, total nitrogen and total phosphorus.

Salamander larvae were captured and handled under the permission of the Lombardy regional administration, P. T1.2009.0016990 decreed on 2009/09/16, and administrative order 964 decreed on 2013/02/11 for 2013–2014.

Biochemical and genetic data

Fire salamander tissues were suspended and homogenised with nine volumes of ice cold Hepes–Tris 10 mM, pH 7.5, containing 50 mM mannitol and 1 mM dithiothreitol. The crude extract was then centrifuged at 15 000 × g (4°C) for 30 min and the supernatant was used to measure the enzyme activity of GST and CAT. Since pesticides could lead to significant oxidative stress responses (Ranjan and Jindal 2022), we measured acetylcholinesterase (AChE) activity to account for their possible confounding effect. We used AChE to control the possible effects of pesticide pollution, as it is recognized to be more sensitive than GST and CAT as a biomarker (Pérez et al. 2004). For the AChE assay, tissue was homogenised in 20 mM sodium phosphate, pH 7.4, containing 250 mM sucrose and 1% Triton X-100, and processed as above. Enzymes were assayed spectrophotometrically as described by Berra et al. (2004). Enzyme activities were performed in duplicate and expressed as specific activity (U mg^{-1}) where protein concentration (mg ml^{-1}) was determined according to Bradford (1976).

Using genotype data obtained from the 16 species-specific microsatellites of Pisa et al. (2015), we estimated the individual inbreeding coefficient (F) through the adegenet package (Jombart and Ahmed 2011) in R (<www.r-project.org>).

To investigate the effect of genetic and environmental factors on enzyme expressions, we associated each sampled individual for which we measured enzyme activities with both water physicochemical parameters and the mean of inbreeding (F) calculated over all genotyped individuals of the corresponding SL (or nearest genetic SL; maximum distance between enzyme and genetic SL less than 300 m).

Statistical analyses

We first tested for the causal effect of habitat fragmentation on inbreeding by correlating the inbreeding of each individual in each SL with the logarithm of the ecological distance with barriers to the nearest SL (i.e. intra-SP isolation), correcting for Euclidean distance (Table S5 and S3 from Bani et al. 2015, respectively). We tested for possible differences in the level of inbreeding among SPs, as well as differences in GST and CAT activity, using an ANOVA, based on the pairwise t-test in R (a square-root transformation was used to obtain a normal distribution of the measures). The same analysis was adopted to explore possible differences in the AChE activity among SPs.

We evaluated the effect of inbreeding on GST and CAT enzyme activity, controlling for water physicochemical parameters and larval stage, using a linear mixed model through the lme4 package (Bates et al. 2015) in R. Based on the structure of the dataset, we used the sampling location as a random factor. The values of all the covariates were scaled (Harrison et al. 2018). We performed a manual backward selection of covariates starting from the one with the lowest absolute t-value, until all covariates in the model were statistically significant ($p < 0.05$).

Results

We found a significant correlation between inbreeding of individuals and fragmentation-induced isolation (Pearson's $r = 0.24$; $p = 0.006$). The ANOVA showed significantly different levels of inbreeding among SPs ($F = 2.792$; $p = 0.029$; Fig. 2a), highlighting how intra-SP isolation can affect inbreeding at the SL level (Bani et al. 2015). Indeed, AG and FC, located in a more continuous landscape, showed a significantly lower level of inbreeding (all $p < 0.05$) than MV and BB, located in a more fragmented context. The level of inbreeding found in CF did not differ significantly from all other SPs.

The ANOVA also showed a significant difference in the GST activity among SPs ($F = 20.62$, $p < 0.001$; Fig. 2b), but not for AChE (Fig. 2c) and CAT (Fig. 2d). GST was significantly lower in AG and FC than in all other SPs (all $p < 0.001$). The highest GST activity was found in BB, which differed significantly from MV ($p < 0.001$).

Regression models showed a significant positive effect of inbreeding level on GST (marginal $\beta = 0.049$, $p = 0.003$; Fig. 3a) The GST model showed good explanatory performance (conditional R^2 0.709, marginal R^2 0.370)

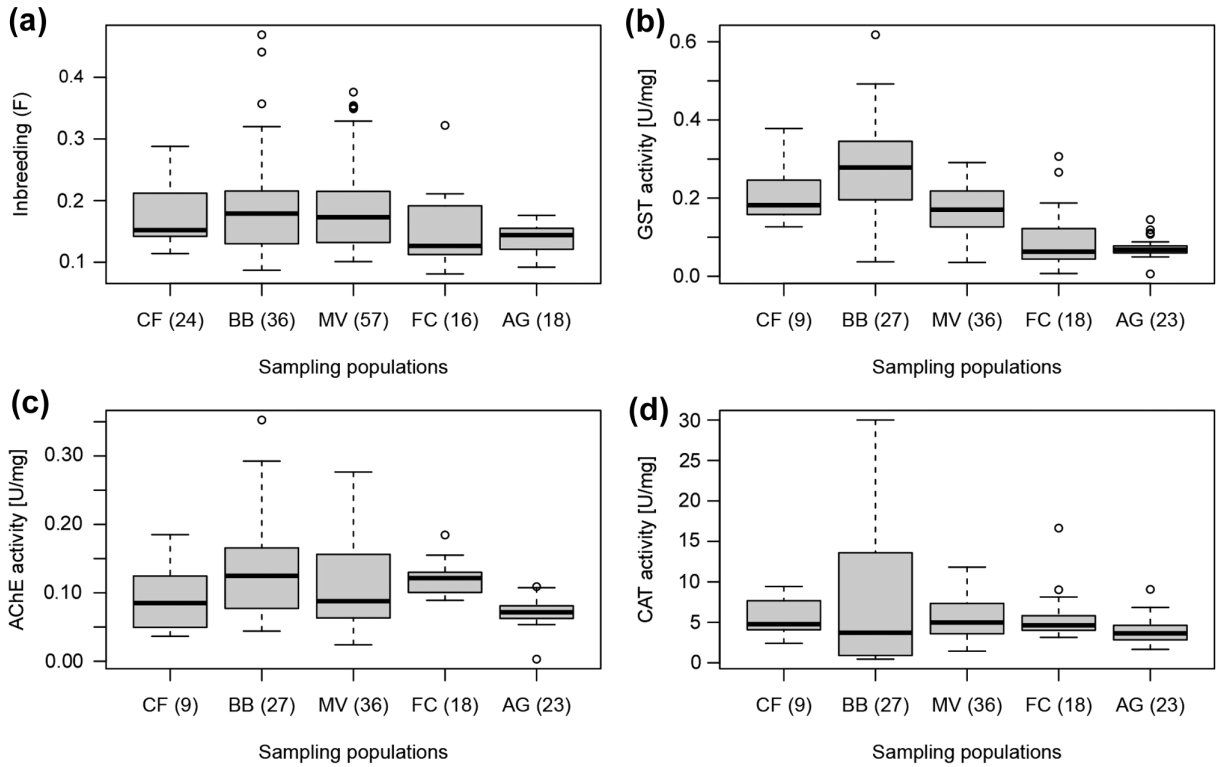

Figure 2. Boxplots of (a) inbreeding estimates (F), (b) GST glutathione S-transferase (GST), (c) acetylcholinesterase (AChE) and (d) catalase (CAT) activities [U mg^{-1}] in the sampling populations. Sample size in brackets.

and, as expected, oxygen saturation (marginal $\beta = 0.044$, $p = 0.009$), total nitrogen (marginal $\beta = -0.047$, $p = 0.006$) and water temperature (marginal $\beta = -0.049$, $p = 0.003$) also played a significant role in GST activity. Conversely, neither the level of inbreeding ($\beta = -0.148$, $p = 0.184$; Fig. 3b) nor any of the environmental covariates affected the CAT activity (it should be noted that, in this case, the β-value indicated for the inbreeding refers to an estimate obtained from an univariate model after the complete step-by-step removal of all environmental covariates that have never resulted significant).

Discussion

Animals exhibit high levels of GST and CAT as a defence against heavy metals or pesticides, to mediate biotransformation of xenobiotics and detoxification from hydrogen peroxide, respectively. Concomitant inhibition of AChE, which causes impairment of synaptic signalling, is a warning of pollution risk (Limón-Pacheco and Gonsebatt 2009, Handy and Loscalzo 2012, Rudneva 2013, Gobi et al. 2018, Stara et al. 2019a, b). The high GST values detected in our studies were probably not due to environmental pollutants, as we did not

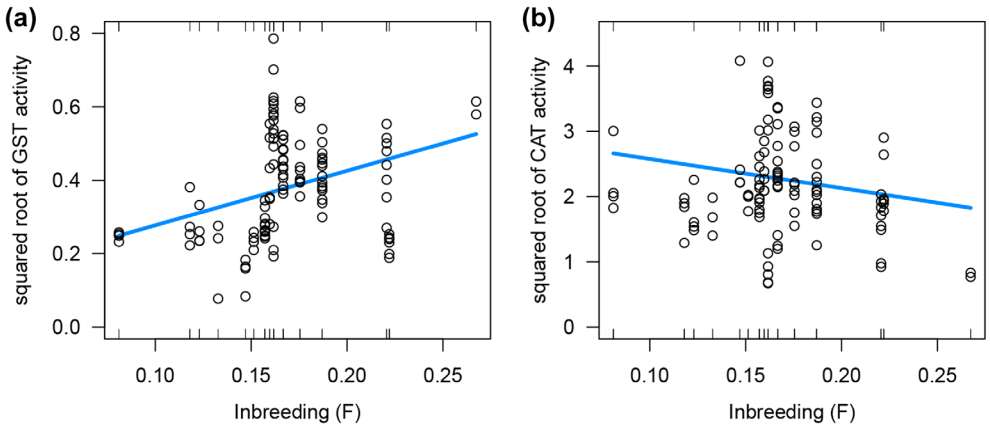

Figure 3. Marginal effect of inbreeding on GST activity (a), and effect of inbreeding on CAT activity (b); see the text for the β values and their significance.

observe concomitant high CAT and low AChE measurements. We also exclude the possible confounding effect of sex in enzyme expression, since it is unlikely that our sample was strongly unbalanced with respect to sex as individuals were randomly sampled from breeding pools.

Our hypothesis is that inbreeding may have negatively impacted aerobic respiration metabolism, leading to ROS overproduction, through an accumulation of mDNA damage across generations (Beekman et al. 2014). We therefore suggest that high GST activity may be symptomatic of oxidative stress derived from inbreeding conditions and strongly encourage further assessment of a broader panel of oxidative biomarkers, including superoxide dismutase and glutathione peroxidase (both stimulated by cell structure damage), to provide more compelling evidence.

Noteworthy, some studies seem to support this hypothesis. A higher frequency in homozygous recessive deleterious alleles causes alterations in homeostasis and may lead to accumulation of misfolded proteins (Kristensen et al. 2010, Hedrick and Garcia-Dorado 2016). Hsp70 has been suggested to work as a buffering system to counteract the increasing demand for chaperones in inbred flies (Kristensen et al. 2002, Pedersen et al. 2005, Reed et al. 2012), where it is highly expressed even during exposure to a benign environment. Genome-wide gene expression and metabolomic analysis showing differential enrichment for elements involved in metabolism and stress responses (Kristensen et al. 2005, Pedersen et al. 2008). Similar observations have been found in the Yesso scallop (Zhao et al. 2019) and the Pacific abalone (Cheng et al. 2006). Comparably, we speculate that GST overexpression might reflect dysregulated cellular activities due to genetic load and represent a protective mechanism to compensate for oxidative unbalance.

However, possible inter-individual discrepancies and the genetic background underlying inter-population differences must also be considered (Costantini et al. 2005, 2009, 2012, Careau et al. 2008, Samaras et al. 2016, Lallias et al. 2017, Raulo and Dantzer 2018, Wong et al. 2019). Therefore, we can also postulate the presence of a pool of individuals carrying a highly expressed form of GST, whose increase in allele frequency is facilitated by conditions that favour inbreeding.

These two interpretations attempt to link GST activity and inbreeding levels from a genetic perspective. An additional third explanation can be argued, pointing more broadly to the ecological consequences of habitat fragmentation: small and isolated habitat fragments tend to reduce the size of home ranges, resulting in increased intra-specific competition for resources or exacerbation of prey-predator interactions. The effect of such stressful events would be reflected in increased GST activity.

All perspectives suggest the possibility that the negative effects of inbreeding-environment interactions may be mediated by a disruption of the oxidative balance. Decreased adaptability to environmental changes would contribute to reduce fitness, providing an explanation for the phenomenon of inbreeding-depression, whose physiological and molecular mechanisms are not clearly understood (Ayroles et al. 2009, Kristensen et al. 2010, Fox and Reed 2011, Reed et al. 2012, Hedrick and Garcia-Dorado 2016). Indeed, inbred fly populations with higher levels of Hsp70 were less tolerant to heat stress (Pedersen et al. 2005, Cheng et al. 2006) and inbred males had testicular oxidative stress and a decreased fertility (Okada et al. 2011). In adult songbirds, the co-occurrence of inbreeding and disadvantageous postnatal conditions resulted in impaired oxidative status (de Boer et al. 2018).

As our data were collected in the field, we would like to stress that other environmental covariates, natural and anthropogenic, not considered in this study may have affected our results and we must therefore be cautious in their interpretation. However, given the significant implications of our findings from both a conservation and health perspective, this research should urgently stimulate further ad hoc studies aimed at confirming or disproving the effect of fragmentation-induced isolation on antioxidant responses. Indeed, the lack of wild population studies focusing on inbreeding and oxidative stress currently makes it difficult to draw well-defined conclusions.

Speculations

Our results provided suggestions that the antioxidant system may not be affected only by physical or chemical alterations. Therefore, the significance of some biomarkers canonically used in the evaluation of environmental stressors should be interpreted more carefully. However, to obtain a more complete picture of the oxidative status of individuals threatened by habitat fragmentation, and to confirm our findings, further studies should include the measurement of more antioxidant parameters (especially SOD, glutathione-dependent system GSH-Px, GSH), as well as other aspects of oxidative stress, such as ROS concentrations and oxidative damage parameters. We emphasize the importance of new studies in this area, as our speculation is that inbreeding-induced oxidative stress may contribute to deteriorating health status of wildlife populations. Indeed, sources of stress can trigger immunosuppressive conditions (Sapolsky et al. 2000, Dhabhar 2009, Uren Webster et al. 2018) and the alteration of the oxidative status may favour infection diseases. Thus, fragmentation-induced isolation can play a key role in increasing animals' sensitivity to environmental stressors and their susceptibility to pathogen infection. In addition, fragmentation leads to a reassembly of local communities with new inter-species contacts. Particular attention should be paid to these emerging interactions as they can generate spillover phenomena and, ultimately, zoonoses (Plowright et al. 2021).

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Author contributions

Luciano Bani: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Funding acquisition (equal); Methodology (equal); Writing – original draft (equal). **Valerio Orioli**: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing – review and editing (equal). **Roberto Giacchini**: Investigation (equal); Methodology (equal); Writing – review and editing (equal). **Paolo Parenti**: Data curation (equal); Validation (equal); Writing – review and editing (equal). **Olivia Dondina**: Methodology (equal); Validation (equal); Writing – review and editing (equal). **Marko Prokić**: Validation (equal); Writing – review and editing (equal). **Caterina Faggio**: Validation (equal); Writing – review and editing (equal). **Giulia Campli**: Conceptualization (equal); Validation (equal); Writing – original draft (equal); Writing – review and editing (equal).

Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.fxpnvx0vm> (Bani et al. 2022).

References

Aliko, V. et al. 2018. Antioxidant defense system, immune response and erythron profile modulation in gold fish, *Carassius auratus*, after acute manganese treatment. – Fish Shellfish Immunol. 76: 101–109.

Allentoft, M. E. and O'Brien, J. 2010. Global amphibian declines, loss of genetic diversity and fitness: a review. – Diversity 2: 47–71.

Ayroles, J. F. et al. 2009. A genomewide assessment of inbreeding depression: gene number, function and mode of action. – Conserv. Biol. 23: 920–930.

Bani, L. et al. 2015. Ecological connectivity assessment in a strongly structured fire salamander *Salamandra salamandra* population. – Ecol. Evol. 5: 3472–3485.

Bani, L. et al. 2022. Data from: Can antioxidant responses be induced by habitat fragmentation process? – Dryad Digital Repository, <https://doi.org/10.5061/dryad.fxpnvx0vm>.

Bates, D. et al. 2015. Fitting linear mixed-effects models using lme4. – J. Stat. Softw. 67: 1–48.

Beaulieu, M. and Costantini, D. 2014. Biomarkers of oxidative status: missing tools in conservation physiology. – Conserv. Physiol. 2: cou014.

Beekman, M. et al. 2014. The costs of being male: are there sex-specific effects of uniparental mitochondrial inheritance? – Phil. Trans. R. Soc. B 369: 20130440.

Berra, E. et al. 2004. Evaluation of enzyme biomarkers in freshwater invertebrates from Taro and Ticino River, Italy. – Ann. Limnol. Int. J. Limnol. 40: 169–180.

Blaustein, A. R. et al. 1994. Amphibian declines: judging stability, persistence and susceptibility of populations to local and global extinctions. – Conserv. Biol. 8: 60–71.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. – Anal. Biochem. 72: 248–254.

Careau, V. et al. 2008. Energy metabolism and animal personality. – Oikos 117: 641–653.

Carlitz, E. H. D. et al. 2016. Measuring hair cortisol concentrations to assess the effect of anthropogenic impacts on wild chimpanzees *Pan troglodytes*. – PLoS One 11: e0151870.

Cheng, P. et al. 2006. Heat-shock protein70 gene expression in four hatchery Pacific abalone *Haliotis discus hannai* Ino populations using for marker-assisted selection. – Aquacult. Res. 37: 1290–1296.

Costantini, D. et al. 2005. Inter-population variation of carotenoids in Galápagos land iguanas *Conolophus subcristatus*. – Comp. Biochem. Physiol. B Biochem. Mol. Biol. 142: 239–244.

Costantini, D. et al. 2009. Temporal and spatial covariation of gender and oxidative stress in the Galápagos land iguana *Conolophus subcristatus*. – Physiol. Biochem. Zool. 82: 430–437.

Costantini, D. et al. 2012. Interplay between plasma oxidative status, cortisol and coping styles in wild alpine marmots, *Marmota marmota*. – J. Exp. Biol. 215: 374–383.

de Boer, R. A. et al. 2018. Sex-specific effects of inbreeding and early life conditions on the adult oxidative balance. – Curr. Zool. 64: 631–639.

Denoël, M. 1996. Phénologie et domaine vital de la salamandre terrestre *Salamandra salamandra* terrestris (Amphibia, Caudata) dans un bois du Pays de Herve (Belgique). – Cahiers d'Ethol. 16: 291–306.

Dhabhar, F. S. 2009. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. – Neuroimmunomodulation 16: 300–317.

Dondina, O. et al. 2018. Ecological network design from occurrence data by simulating species perception of the landscape. – Landscape Ecol. 33: 275–287.

Dondina, O. et al. 2019. Species specialization limits movement ability and shapes ecological networks: the case study of 2 forest mammals. – Curr. Zool. 65: 237–249.

Fox, C. W. and Reed, D. H. 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. – Evol. Int. J. Org. Evol. 65: 246–258.

Gobi, N. et al. 2018. Bioaccumulation, cytotoxicity and oxidative stress of the acute exposure selenium in *Oreochromis mossambicus*. – Ecotoxicol. Environ. Safety 162: 147–159.

Halliwell, B. and Gutteridge, J. M. C. 2015. Free radicals in biology and medicine. – Oxford Univ. Press.

Handy, D. E. and Loscalzo, J. 2012. Redox regulation of mitochondrial function. – Antioxid. Redox Signal. 16: 1323–1367.

Hanski, I. and Simberloff, D. 1997. The metapopulation approach, its history, conceptual domain and application to conservation. – In: Hanski, I. and Gilpin, M. E. (eds), Metapopulation biology. Academic Press, pp. 5–26.

Harrison, X. A. et al. 2018. A brief introduction to mixed effects modelling and multi-model inference in ecology. – PeerJ 6: e4794.

Hedrick, P. W. and Garcia-Dorado, A. 2016. Understanding inbreeding depression, purging and genetic rescue. – Trends Ecol. Evol. 31: 940–952.

Johnstone, C. P. et al. 2012. Does habitat fragmentation cause stress in the agile antechinus? A haematological approach. – J. Comp. Physiol. B 182: 139–155.

Jombart, T. and Ahmed, I. 2011. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. – Bioinformatics 27: 3070–3071.
Kristensen, T. N. et al. 2002. Inbreeding affects Hsp70 expression in two species of *Drosophila* even at benign temperatures. – Evol. Ecol. Res. 4: 1209–1216.
Kristensen, T. N. et al. 2005. Genome-wide analysis on inbreeding effects on gene expression in *Drosophila melanogaster*. – Genetics 171: 157–167.
Kristensen, T. N. et al. 2010. Research on inbreeding in the 'omic' era. – Trends Ecol. Evol. 25: 44–52.
Kurutas, E. B. 2015. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. – Nutr. J. 15: 1–22.
Lallias, D. et al. 2017. Genetic variability of environmental sensitivity revealed by phenotypic variation in body weight and (its) correlations to physiological and behavioral traits. – PLoS One 12: e0189943.
Limón-Pacheco, J. and Gonsebatt, M. E. 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. – Mutat. Res./ Genet. Toxicol. Environ. Mutagen. 674: 137–147.
Lowe, W. H. and Allendorf, F. W. 2010. What can genetics tell us about population connectivity? – Mol. Ecol. 19: 3038–3051.
Martínez-Mota, R. et al. 2007. Effects of forest fragmentation on the physiological stress response of black howler monkeys. – Anim. Conserv. 10: 374–379.
Moore, J. A. et al. 2011. Effects of the landscape on boreal toad gene flow: does the pattern–process relationship hold true across distinct landscapes at the northern range margin? – Mol. Ecol. 20: 4858–4869.
Okada, K. et al. 2011. Male attractiveness, fertility and susceptibility to oxidative stress are influenced by inbreeding in *Drosophila simulans*. – J. Evol. Biol. 24: 363–371.
Pedersen, K. S. et al. 2005. Effects of inbreeding and rate of inbreeding in *Drosophila melanogaster*-Hsp70 expression and fitness. – J. Evol. Biol. 18: 756–762.
Pedersen, K. S. et al. 2008. Metabolomic signatures of inbreeding at benign and stressful temperatures in *Drosophila melanogaster*. – Genetics 180: 1233–1243.
Pérez, E. et al. 2004. Biomarker responses to pollution in two invertebrate species: *Scrobicularia plana* and *Nereis diversicolor* from the Cádiz bay (SW Spain). – Mar. Environ. Res. 58: 275–279.
Pisa, G. et al. 2015. Detecting a hierarchical genetic population structure: the case study of the fire salamander *Salamandra salamandra* in Northern Italy. – Ecol. Evol. 5: 743–758.
Plowright, R. et al. 2021. Land use-induced spillover: a call to action to safeguard environmental, animal and human health. – Lancet Planet. Health 5: e237–e245.
Rainio, M. J. et al. 2013. Oxidative status in nestlings of three small passerine species exposed to metal pollution. – Sci. Total Environ. 454: 466–473.
Ranjan, A. and Jindal, T. 2022. Toxicology of organophosphate poisoning. – Springer Nature.
Raulo, A. and Dantzer, B. 2018. Associations between glucocorticoids and sociality across a continuum of vertebrate social behavior. – Ecol. Evol. 8: 7697–7716.
Reed, D. H. et al. 2012. Inbreeding–stress interactions: evolutionary and conservation consequences. – Ann. N. Y. Acad. Sci. 1256: 33–48.
Rudneva, I. 2013. Biomarkers for stress in fish embryos and larvae. – CRC Press.
Samaras, A. et al. 2016. Repeatability of cortisol stress response in the European sea bass *Dicentrarchus labrax* and transcription differences between individuals with divergent responses. – Sci. Rep. 6: 34858.
Sapolsky, R. M. et al. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory and preparative actions. – Endocr. Rev. 21: 55–89.
Schmidt, B. R. et al. 2005. Demographic processes underlying population growth and decline in *Salamandra salamandra*. – Conserv. Biol. 19: 1149–1156.
Schulte, U. et al. 2007. A PIT tag based analysis of annual movement patterns of adult fire salamanders *Salamandra salamandra* in a Middle European habitat. – Amphibia-Reptilia 28: 531–536.
Schweizer, M. et al. 2019. Snails in the sun: strategies of terrestrial gastropods to cope with hot and dry conditions. – Ecol. Evol. 9: 12940–12960.
Sies, H. 1986. Biochemistry of oxidative stress. – Angew. Chem. Int. Ed. Engl. 25: 1058–1071.
Stara, A. et al. 2019a. Acute exposure of common yabby *Cherax destructor* to the neonicotinoid pesticide. – Sci. Total Environ. 665: 718–723.
Stara, A. et al. 2019b. Effects of S-metolachlor and its degradation product metolachlor OA on marbled crayfish *Procambarus virginalis*. – Chemosphere 224: 616–625.
Suorsa, P. et al. 2003. Forest management is associated with physiological stress in an old-growth forest passerine. – Proc. R. Soc. B 270: 963–969.
Tischendorf, L. and Fahrig, L. 2000. On the usage and measurement of landscape connectivity. – Oikos 90: 7–19.
Uren Webster, T. M. et al. 2018. Contrasting effects of acute and chronic stress on the transcriptome, epigenome and immune response of Atlantic salmon. – Epigenetics 13: 1191–1207.
Vinagre, C. et al. 2012. Effect of temperature on oxidative stress in fish: lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. – Ecol. Indic. 23: 274–279.
Wong, R. Y. et al. 2019. Differences in stress reactivity between zebrafish with alternative stress coping styles. – R. Soc. Open Sci. 6: 181797.
Zhao, L. et al. 2019. Transcriptomic profiling provides insights into inbreeding depression in Yesso scallop *Patinopecten yessoensis*. – Mar. Biotechnol. 21: 623–633.