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BEHAVIOURAL ECOLOGY OF A LATE QUATERNARY RANGE EXPANSION

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OUTLINE OF THE THESIS

This thesis is based on the study of the evolutionary and biogeographic outcomes that historical expansions may have on animal populations. In Chapter 1 the background, the general aims of the research as well as the historical biogeographic context and the study species are presented. Chapter 2 introduced the study of personality traits, the results obtained and their discussion. In Chapter 3 the study of locomotory performance traits is presented, and the results obtained are showed and discussed. In Chapter 4 the study of oxidative status physiology is introduced, and the results obtained are presented and discussed. Chapter 5 introduced the study of telomere dynamics, the results obtained and their discussion. A general discussion of the results obtained and of their relevance in the light of the general aim of the research is reported in Chapter 6. The cited literature is presented in in the last section of the thesis.

Finally, two manuscripts submitted to scientific journals are presented in the appendix.

CHAPTER 1 BACKGROUND OF RESEARCH

Dispersal is defined as any movement of individuals away from their point of origin (Brown and Lomolino, 1998). It is possible to distinguish two spatial patterns of dispersal: diffusion and jump dispersal (Wilkinson, 2017). Diffusion implies a slowly expanding population starting from the edge of its geographic range, while jump dispersal occurs when a small number of individuals disperse to a distant location from the edge of their current range (Wilkinson, 2017). Dispersal affects significantly the ecological and evolutionary dynamics of animal populations (Ronce, 2007; Burton et al., 2010; Clobert et al., 2012; Travis et al., 2013; Bonte and Dahirel, 2017; Cote et al., 2017; Legrand et al., 2017) because of its consequences for demography and gene flow (Hanski and Gilpin, 1991; Hansson, 1991; Bohonak, 1999; Clobert, 2001; Saastamoinen et al., 2018).

Dispersal has a substantial within species variation and mounting evidence suggests that dispersers are a not a random subset of a population's phenotypic pool (Bowler and Benton, 2005; Clobert et al., 2009; Lowe et al., 2014; Cote et al., 2017). Instead, dispersers are those individuals who, compared to the average of the entire population, carry a suite of traits that enhances their dispersal propensity and ability. Individual differences in dispersal propensity have been associated with variation in several phenotypic traits including personality (e.g. Fraser et al., 2001; Cote et al., 2010a), locomotion (Phillips et al., 2006, 2008; Louppe et al., 2017), morphology (Forsmann et al., 2010; Shine et al., 2011; Hudson et al., 2016a), or physiology (Liebl and Martin, 2013; Selechnik et al., 2017). In the course of an expansion, the evolutionary and ecological pressures should select the best dispersers, leading to an increasing frequency of the phenotypic traits associated with them (spatial sorting) along the expansion axis (Simmons and Thomas, 2004; Phillips et al., 2010; Shine et al., 2011; Canestrelli et al., 2016b). This process, can drive a deterministic and, often rapid, evolution of the phenotypic make up of expanding populations (Travis and Dytham, 2002; Phillips et al., 2010; Shine et al.,

2011). Divergent phenotypes are indeed observed across the range of several expanding populations between individuals from native and invaded areas and also between individuals from recently invaded areas and those from past invaded areas (Phillips et al., 2006; Cote et al., 2010b; Chapple et al., 2012; Liebl and Martin, 2012; Brown et al., 2015; Courant et al., 2017; Gruber et al., 2017a; Kosmala et al., 2017).

A nice exemplification of the rapid and deterministic evolution of the phenotypic makeup in an expanding population is that of the cane toad (*Rhinella marina*), a highly successful invasive species in Australia, where it spread at increasing rate for some 80 years (Phillips et al., 2006, 2010). It has been extensively documented a rapid evolution in dispersal enhancing traits across the invaded range. Toads at the invasion front grow faster, have longer legs and more compact skull, move more often, for longer times and with straighter paths than those living in past invaded areas (Phillips et al., 2006, 2008, 2009, 2010; Lindström et al., 2013; Brown, Phillips and Shine, 2014; Hudson et al., 2016a). Moreover, at the invasion front, the locomotor endurance is higher (Llewellyn et al., 2010), toads are bolder and more explorative (Gruber et al., 2017a), show different immunological profiles (Brown and Shine, 2014; Brown et al., 2015) and genes associated with metabolism and cellular damage repair are upregulated compared to the rest of the population (Rollins et al., 2015).

The ongoing biological invasions provide an opportunity to study the deterministic changes in the phenotypic makeup of an expanding populations. However, the study of ongoing processes makes difficult the investigation of their biogeographic and evolutionary outcomes (Canestrelli et al., 2016b). In fact, as any ongoing expansion process is transient by definition, the selective advantages associated with adaptive traits during this phase should be transient, as well. It is indeed hypothesized that at the end of an expansion event, the recently established population may evolve towards a condition of demographic equilibrium by recovering variation (phenotypic and genotypic) through a slower flow of individuals from the back (Cobben et al., 2015; Canestrelli et al., 2016a).

To date, no studies have investigated if the directional changes in dispersal-enhancing traits as a consequence of historical expansion processes persist. It is, therefore, completely unknown if and how the outcomes of historical expansions may have played a role in shaping the current biogeographic patterns of species phenotypic variation.

The Late Quaternary range expansions are considered to be the most important historical processes in structuring the current species biogeography (Taberlet et al., 1998; Hewitt, 2000; Thompson, 2005). In particular, as a consequence of Pleistocene glacial-interglacial cycles, several temperate species have repeatedly faced contractions and expansions of their ranges (Hewitt, 1996, 1999, 2000; Taberlet et al., 1998; Weiss and Ferrand, 2007).

One temperate species that underwent a range expansion during the Late Quaternary is the Tyrrhenian tree frog (*Hyla sarda*). The evolutionary history of this species represents a unique model to investigate the directional changes in dispersal-enhancing traits as a consequence of historical range expansion. The species expanded from Sardinia to Corsica up to colonize the Tuscan Archipelago during the last glaciation (Bisconti et al., 2011b). The diffusion from Sardinia to Corsica was permitted by the temporary emersion, as a result of marine regressions, of a wide land-bridge connecting the two islands (Van Andel and Shackleton, 1982; Shackleton et al., 1984). The marine transgressions that followed the expansion, would have isolated again the two islands thus closing the access to Corsica. This would have interrupted any possibility of further flow of individuals from the back, preventing the loss of the evolutionary legacy of the previous expansion phase. On the other hand, the absence of any connection between Corsica and Elba island suggest that the latter was probably colonized through a jump overseas dispersal (Vences et al., 2003, 2004).

In the present study, I evaluate for the first time if a historical expansion may have acted as deterministic “generator” of disperser profiles. In other words, I search for a phenotypic legacy as a consequence of a historical range expansion. More specifically, I investigate whether, in the context of Late Quaternary expansions, dispersal dynamics may have contributed to shape

the geographic patterns in phenotypic variation. To achieve this goal, I characterized the geographical variation of four categories of traits potentially implicated in dispersal dynamics along the inferred route of the *H. sarda* historical expansion (Bisconti et al., 2011b). I characterized the geographic variation of behavioural (personality traits), locomotory (jumping performance) and physiological (oxidative status) traits. Several evidences indeed suggest that these traits are key components of dispersal propensity (Dingemanse et al., 2003; Phillips et al., 2006; Liebl and Martin, 2013; Rollins et al., 2015; Gruber et al., 2017a; Louppe et al., 2017; Selechnik et al., 2017) and therefore could have been under strong selection during historical expansion processes. Moreover, as telomere length variation has been implicated in processes of ecological and evolutionary importance in a wide range of organisms (Ilmonen et al., 2008; Angelier et al., 2013; Hau et al., 2015; Adriaenssens et al., 2016), I investigated whether the biogeographic history, a key driver of eco-evolutionary processes, may have promoted the evolution of telomere dynamics.

1.1 The Quaternary expansions

Paleoclimate has deeply influenced the current worldwide species distribution (Hewitt, 2000; Hewitt, 2004; Schmitt, 2007). A key biogeographic role was played by the Pleistocene climatic fluctuations (Hays et al., 1976; Hewitt, 1996; Hofreiter and Stewart, 2009) during which the glacial-interglacial cycles occurred with different consequences for species distributions across the globe (Hewitt, 2004).

In Europe, the geographic structure of landscape underwent rapid transformations in response to such massive climate changes (Van Andel and Tzedakis, 1996). This new geography has greatly influenced the distribution of many species, which have faced continuous contractions and expansions of their ranges as shown by phylogeographic reconstructions (Hewitt, 1996, 1999, 2000; Taberlet et al., 1998; Weiss and Ferrand, 2007). On the continent, several temperate species have survived the hostile glacial conditions by contracting their range in suitable areas located in the southern peninsulas of Iberia, Italy and the Balkans (Taberlet et al., 1998; Hewitt, 2000; Stewart and Lister, 2001; Feliner, 2001; Schmitt, 2007; Bennet and Provan, 2008; Stewart et al., 2010) while during the interglacial phases, they would have experienced range expansion in response to more permissive climatic conditions, by re-colonizing the northern habitats (Taberlet et al., 1998; Hewitt, 1999).

On islands, a different scenario occurred. Recent studies revealed a pattern of glacial expansion and interglacial contraction, at least for some species endemic to Mediterranean islands (Bisconti et al., 2011a; Salvi et al., 2014; Senczuk et al., 2019). This scenario is explained by the Pleistocene sea level oscillations that strongly affected the paleogeography of the Mediterranean Sea (Van Andel and Shackleton, 1982; Shackleton et al., 1984), as well as the evolutionary history of species living there (Thompson, 2005; Papadopoulou et al., 2009; Lázaro et al., 2011; Bisconti et al., 2011a; Senczuk et al., 2019). In particular, during the last glacial maximum (LGM, about 23 000–19 000 before present) the sea level dropped up to 120

meters below the current level, leading to a significant seabed emersion (Vogiatzakis et al., 2008). Coastal lowlands increased in extension and different islands were connected between them and with the continent (Van Andel and Shackleton, 1982; Shackleton et al., 1984), creating transit corridors for species (Hewitt, 2011a). Low-altitude areas (0-500 m above the sea level) were 50% wider during the LGM than before (based on data from Jarvis et al., 2008). Moreover, on islands, the adverse glacial climate would have been mitigated by the sea, ensuring favourable environmental conditions (Cronk, 1997; Whittaker and Fernández-Palacios, 2007). Many evidences suggest how these paleoenvironmental changes would have balanced the negative demographic effects of glacial climate (Canestrelli et al., 2007; Canestrelli et al., 2008; Buckley et al., 2009; Porretta et al., 2011). In fact, coastal lowlands have been identified as the hospitable glacial refugia for temperate species around the world (Burns et al., 2007; Marske et al., 2009; Bisconti et al., 2011b; Leite et al., 2016; Senczuk et al., 2019). Possibly, such increase in coastal lowlands exerted its major effect on lowland-adapted species (Bisconti et al., 2011a; Salvi et al., 2014; Senczuk et al., 2019).

1.2 The study species: why *Hyla sarda*?

The study species is the Tyrrhenian tree frog, *Hyla sarda* (De Betta, 1853), a species of the family *Hylidae* (Figure 1) endemic to Sardinia, Corsica and Tuscan Archipelago (Western Mediterranean Sea). The species was chosen because it has unique characteristics allowing to test hypotheses on the phenotypic legacy from historical expansion that includes both the spatial diffusion from Sardinia to Corsica and the colonization of the islands of the Tuscan Archipelago trough jump dispersal.

Figure 1 Study species *Hyla sarda* on a log. Photo credits (Wikimedia Commons) author/hu:User:Ineptus

First, its evolutionary history and historical demography have been already inferred on the basis of nuclear and mitochondrial markers diversity (Bisconti et al., 2011b). Two main clades were

identified, the clade South, in a restricted area of south-eastern Sardinia, and the clade North, distributed in the central-northern area of Sardinia and in the rest of the species' range (Corsica and Tuscan Archipelago). The geographical distribution, as well as the structure of genetic diversity of the northern clade, have been interpreted as the result of an expansion event during the last glacial period from northern Sardinia to Corsica up to colonize the Elba island in the Tuscan Archipelago (Bisconti et al., 2011b). This hypothesis is corroborated by the historical demography revealing that *H. sarda* populations were in a marked demographic growth during the last glacial period (Bisconti et al., 2011b). Moreover, the emergence of coastal lowlands during the last glaciation (Van Andel and Shackleton, 1982; Shackleton et al., 1984) might have provided conditions for glacial expansions, making the hypothesis of the *H. sarda* expansion from Sardinia up to Corsica (Bisconti et al., 2011a, b) even more plausible. In contrast, the Elba island was probably colonized through a jump dispersal starting from Corsica, likely occurred through rare overseas dispersal (Vences et al., 2003, 2004). In fact, in spite of the close vicinity, the Elba island was never connected to Corsica while it was certainly connected to the Italian peninsula (e.g. Tortora et al., 2001). This picture is supported by the low genetic diversity of the Elba tree frogs, as compared to the rest of the species' range, as well as by its geographic location at the northern edge of the species' range (Bisconti et al., 2011b).

Second, the species' evolutionary history allows to resolve the transience that characterizes any ongoing expansion process. At the end of an expansion phase, it is indeed hypothesized that the recently established population may evolve towards a condition of demographic equilibrium by recovering variation (phenotypic and genotypic) through a slower flow of individuals from the back (Cobben et al., 2015; Canestrelli et al., 2016a). This dynamic could "dilute" or cancel the legacy of the evolutionary processes that occurred during the previous expansion phase. The peculiar evolutionary history of the *H. sarda* allowed to eliminate the confounding effect of gene flow from individuals from the back. In fact, the species would have expanded from Sardinia up to Corsica during the last glaciation (Bisconti et al., 2011b) when, as a result of

marine regressions, the two islands were united by a wide land-bridge to form a single system (Van Andel and Shackleton, 1982; Shackleton et al., 1984). The marine transgressions that followed the expansion would have isolated again the two islands thus closing the access to Corsica. This would have interrupted any possibility of further flow of individuals from the back.

Third, the species occurs mainly in coastal areas, which following an assessment of current and peri-glacial bioclimatic suitability, resulted substantially homogeneous, both spatially and temporally (Bisconti et al., 2011b). This allowed to control for local climatic effects potentially affecting the geographic patterns of the investigated traits.

Fourth, the species is abundant throughout its geographical range, with a significant advantage of data collection operations.

CHAPTER 2 PERSONALITY TRAITS

2.1 Introduction

Individuals across many animal species differ consistently in behaviour over time and across multiple contexts. This relatively stable differences in behaviour are defined as animal personality (Réale et al., 2007; Carere and Mastripietri, 2013). Animal personality can influence a wide range of population-level processes with significant ecological and evolutionary implications (e.g. Sih et al., 2004; Réale et al., 2010; Cote et al., 2010a; Wolf and Weissing, 2012; Carere and Maestripieri, 2013; Canestrelli et al., 2016a). In particular, several theoretical studies suggest that some personality traits, such as exploration, boldness and activity may be related with dispersal propensity (Cote et al., 2010a; Chapple et al., 2012; Sih et al., 2012). This prediction has recently stimulated extensive research on dispersal-enhancing personality traits (Cote et al., 2010a and references therein). Indeed, boldness, activity, exploration and aggression have been associated to dispersal propensity in almost all *taxa* (invertebrates: Pintor et al., 2008; Brodin and Drotz, 2014; Hudina et al., 2014; fish: Fraser et al., 2001; Cote et al., 2011; Chapman et al., 2011; Thorlacius, et al., 2015; amphibians: Maes et al., 2012; Brodin et al., 2013; Gruber et al., 2017a; reptiles: Cote and Clobert, 2007; Meylan et al., 2009; Chapple et al., 2011; birds: Verbeek et al., 1994; Dingemanse et al., 2003; Duckworth and Badyaev, 2007; Liebl and Martin, 2012; and mammals: O'Rian et al., 1996; Kracow, 2003; Blumstein et al., 2009). These traits are predicted to differently influence all dispersal stages (i.e. departure, transience, and settlement; Dingemanse et al., 2003; Cote and Clobert, 2007; Cote et al., 2010b).

Boldness is defined as the propensity of an individual to take risks (Réale et al., 2007). Bold individuals are those willing to accept the intrinsic risks of dispersal (Stamps, 2001; Bonte et al., 2012) and thus expected to disperse more frequently than shyer conspecific.

Activity may promote dispersal by enhancing the probability to depart. However, in mole rats (*Heterocephalus glaber*), it has been observed that individuals have also higher locomotor and feeding activities after dispersal (O'Riain et al., 1996). The exploration may also be crucial in promoting dispersal as it potentially allows dispersers to have a greater access to resources, such as water, food, or reproductive partners. In great tits (*Parus major*) it has been observed that natal dispersal distance was associated to exploratory behaviour and that immigrants were faster explorers than resident individuals (Dingemanse et al., 2003). Similarly, aggressiveness may favour dispersers by promoting resources acquisition in new territories. In the western bluebirds (*Sialia mexicana*), more aggressive individuals have a high dispersal propensity allowing them to colonize new habitats and displace its native competitors, the mountain bluebirds (*Sialia currucoides*) (Duckworth, 2006; Duckworth and Badyaev, 2007).

Nowadays, it is widely accepted that dispersers are a non-random sample of a population because they are characterized by a specific suite of personality traits (Bowler and Benton, 2005; Clobert et al., 2009; Cote et al., 2017). Moreover, a growing body of evidence suggests that the geographically variable evolutionary and ecological pressures experienced by any expanding population can drive the directional evolution of individuals at the range-edge (Simmons and Thomas, 2004; Phillips et al., 2010; Shine et al., 2011). In fact, a rapid evolution of divergent personality traits has been observed across the range of several expanding populations. In particular, individuals from range-edge are more exploratory and bolder than those living in long established areas in signal crayfish (*Pacifastacus leniusculus*: Pintor et al., 2008), round goby (*Neogobius melanostomus*: Myles-Gonzalez et al., 2015), cane toad (*Rhinella marina*: Gruber et al., 2017a), common frog (*Rana temporaria*: Brodin et al., 2013), dark-eyed junco (*Junco hyemalis*: Atwell et al., 2012) and house sparrow (*Passer domesticus*: Liebl and Martin, 2012). Individuals from range-edge populations show also more aggression (Duckworth, 2008; Duckworth and Kruuk, 2009) and higher activity (Aragon, 2006a) compared to those from range-core populations. These studies have suggested that personalities

might play an especially critical role in determining the outcome of expansion processes and, thereby, current geographic patterns of species distribution (Foster, 1999; Fraser et al., 2001; Shine et al., 2011; Canestrelli et al., 2016a). Moreover, theoretical models predict that dispersal-enhancing personality (and other such as locomotion and physiology,) traits might persist after a range expansion process ended (Phillips et al., 2010; Shine et al., 2011), as supported by numerous evidences coming from invasive species (Gruber et al., 2017a; Cote et al., 2017). However, the biogeographic and evolutionary consequences of these effects on animal populations remain substantially unexplored (Canestrelli et al., 2016a).

Here, I test weather a past expansion event may have induced a differentiation in personality traits among three islands (Sardinia, Corsica and Elba island) distributed along the inferred route of the *H. sarda* past expansion (Bisconti et al., 2011b). My main hypothesis is that, if individual personality might play an especially critical role in determining the outcome of expansion processes, a spatial structure in personality traits might emerge along the range of a recently expanded population.

I used standardised tests to characterize three personality traits possibly related to dispersal-propensity in this species: spontaneous behaviour, exploration and boldness. The spontaneous behaviour was measured by visual scan sampling in a familiar environment (Réale et al., 2007). exploration was measured with a novel environment test (Réale et al., 2007), during which four response variables, all related to the levels and type of activity, were recorded. Boldness was measured with a "shelter test" by recording the latency to emerge from a shelter into a novel environment (Yoshida et al., 2005; Brown et al., 2007; Cote et al., 2010a). A short latency is commonly associated with a bold personality with a rapid assumption of risks that could derive from an unknown environment (Burns, 2008; Beckmann and Biro, 2013; Carter et al., 2013; Toms et al., 2010).

I compare the geographic variation of the investigated traits between Sardinia and Corsica and between Corsica and Elba island separately. To test a personality differentiation as a

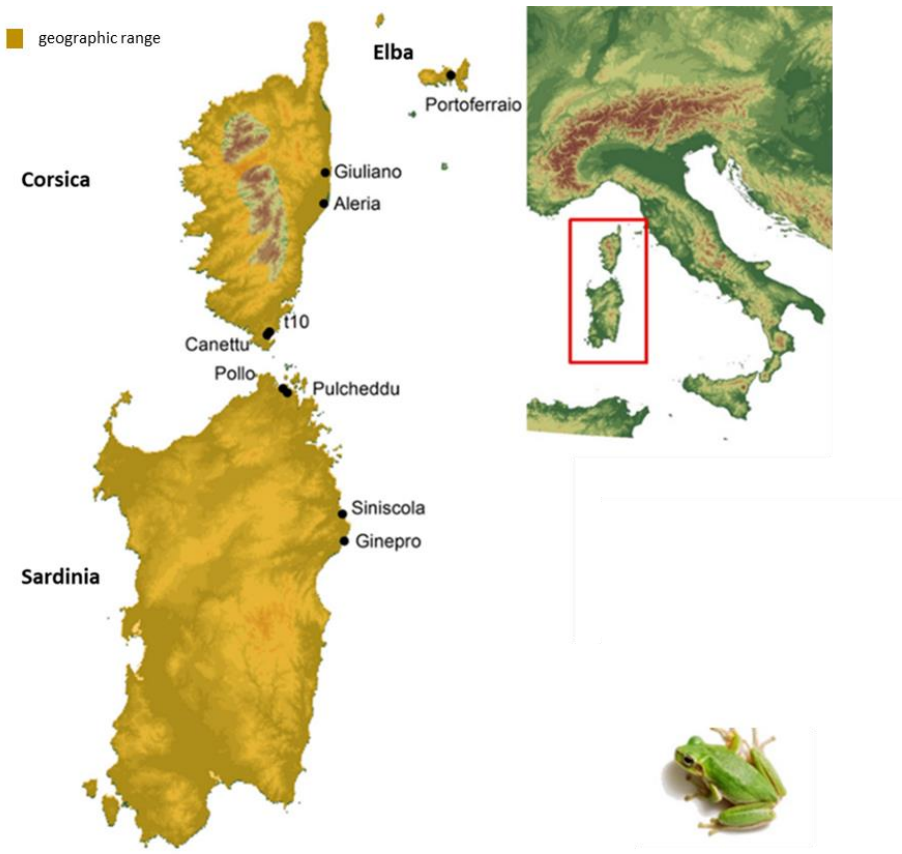
consequence of a Pleistocene range expansion I compare Sardinia with Corsica while, I investigated the outcomes of a past island colonization trough jump dispersal by comparing the same traits between Corsica and Elba island.

2.2 Materials and Methods

2.2.1 Sampling and housing

I carried out sampling along a latitudinal transect from the putative area of glacial refuge in the central-eastern Sardinia to the northern limit of the species current distribution (Elba island) as shown in Figure 1. I sampled a total of 130 individuals of *H. sarda* from five different populations: two in Sardinia, two in Corsica and one in the Elba island. For each population, I collected 20-25 individuals from a minimum of two different locations at a mean distance of about 50 km from each other.

Figure 1 Geographic range of the *Hyla sarda* and geographic distribution of all sampling locations.



All sampling locations were in coastal areas which, following an assessment of current and peri-glacial bioclimatic suitability resulted substantially homogeneous, both spatially and temporally (Bisconti et al., 2011b). This allowed to control for local climatic effects potentially affecting the geographic patterns of the investigated traits. A summary of the geographic coordinates and sample size of all sampled locations are reported in Table 1.

Table 1 Geographic coordinates and sample size of all sampling populations: SS=South Sardinia; NS=North Sardinia; SC=South Corsica; NC=North Corsica; EL Elba.

Island	Population	Location	Lat	Long	N
Sardinia	SS	Cala Ginepro	40.448117	9.792004	19
		Siniscola	40.581462	9.769105	9
	NS	Pulcheddu	41.163977	9.362055	12
		Porto Pollo	41.184479	9.330305	17
Corsica	SC	Canettu	41.445618	9.203529	10
		T10	41.460346	9.217002	18
	NC	Aleria	42.113252	9.522122	11
		San Giuliano	42.267744	9.518411	12
Elba	EL	Portoferraio	42.790183	10.353373	22

I collected individuals with hand nets at night after their acoustic and visual localization and after that, I housed them at CISMAR (Ichthyogenic Experimental Marine Centre) at the Tuscia University (Italy, Tarquinia). Here, I housed individuals in a climatically controlled room at a temperature of 24/25° C, relative humidity of 60-80 % and natural photoperiod. I kept frogs in individual cages (25 cm × 25 cm × 25 cm) provided with a small dechlorinated water tank (diameter 5 cm), a plant *(Epipremnum aureum*) and an oak wood as shelter. The cages were not visually or acoustically isolated from each other and I placed it randomly respect to population of origin. Twice a week I cleaned the cages, I renewed water and I fed the animals with crickets (*Acheta domestica*) of medium size (10-15 mm). Before starting the experimentation, I left individuals undisturbed for two weeks except for routine feeding and cleaning duties.

2.2.2 Ethical note

Sampling procedures were performed under the approval of the Institute for Environmental Protection and Research 'ISPRA' (protocol #5944), Ministry of Environment 'MATTM' (protocol #8275), Regione Sardegna (#12144) and Prefecture of Corsica (#2A20180206002 and #2B20180206001).

Permission to temporarily house amphibians at CISMAR was granted by A.S.L. Tarquinia (Local Health and Veterinary Centre) with license code 050VT427. All handling procedures outlined in the present study were approved by the Ethical Committee of the Tuscia University for the use of live animals. During captivity the animals were monitored daily. No adverse effects on the overall health condition of the tree frogs were observed during the procedures. The animals were released in the original sampling locations at the end of the experimentation.

2.2.3 Behavioural tests

I performed three tests in the following order: (i) spontaneous behaviour; (ii) novel environment test; (iii) shelter test. I used a total of 106 individuals to each test, with an interval of 25 days among each behavioural test. I repeated the novel environment test and the shelter test after 10 days to assess the individual consistency over time. I carried out all tests in the housing room to avoid any change in humidity and temperature conditions potentially affecting the measured behavioural traits (Duellman and Trueb, 1994).

2.2.3.1 Spontaneous behaviour

I measured the spontaneous behaviour in the home cage (Réale et al., 2007) using a visual scan sampling of each individual 6 times daily (9:00, 12:00, 15:00, 18:00, 21:00, 24:00) for two days. The scan sampling covered the whole day because, in a crepuscular species, activity

generally increases during the night. I used a handy audio recorder (zoom H4n Pro) to minimize any possible disturbance. I measured two response variables for each frog: (i) number of inactivity events (hereafter inactivity) defined as any discrete inactivity event longer than 3 s and, measured as the proportion on total scans number. Inactivity differences may indicate a different spontaneous behaviour especially in a cryptic species. (ii) Number of inactivity events on a green substrate (i.e. leaves; hereafter "inactivity on green") defined as any discrete inactivity events on a green substrate longer than 3 s and, measured as the proportion on total number of inactivity events. The colour of a resting substrate could be linked to the ability to camouflage (Stevens and Merilaita, 2009) through background matching characterizing many amphibian's species (Rudh, and Qvarnström, 2013; Kang et al., 2016). I then collected this variable to assess any potential chromatic preferences in the resting substrate under controlled conditions.

2.2.3.2 Novel environment test

I conducted the novel environment test to measure the exploration/avoidance behavioural axis (Réale et al., 2007). Considering the arboreal life style of the *H. sarda*, I used a cylindric arena with vertical as well as horizontal dimension (diameter 145 cm x height 90 cm) enriched with a plant (*Epipremnum aureum*) and an oakwood as shelter of about 8 cm length. This enrichment provided the opportunity to hide thus avoiding that the recorded behaviours could reflect abnormal fear or anxiety rather than a spontaneous exploratory behaviour (Walsh and Cummins 1976; Carter et al., 2013; Kelleher et al., 2018). I gently transferred each individualfrom its home cage onto a jar in the arena periphery. I placed over the jar an overturned plastic cap with a 50 g weight attached to the top for an acclimation period of 2 minutes (Beckmann and Biro, 2013). After the acclimation period was ended, I opened the jar allowing the frog to explore the arena for 10 minutes. After that, I immediately transferred back each individual to its home

cage. To remove any chemical cues or secretion left by previous individuals, I cleaned the arena with dechlorinated water. I tested individuals randomly respect to the population of origin on the same day between 9:00 and 14:00. I filmed all tests with a HD video camera (Nova Germany model DVR AHD-7908) suspended 50 cm above the arena.

Later, I analysed the individual exploration behaviour by using the software Boris 5.1.3 (Friard and Gamba, 2016). I extracted the following variables for each individual: (i) latency to first exposure (hereafter latency), defined as the time needed to each individual to enter the arena. This variable may reflect decision-making time needed to start the exploration in a novel environment, possibly revealing a different exploration strategy among islands. In fact, a longer decision-making time may allow a more careful assessment of the surrounding with a significant reduction of the intrinsic risks associated to the exploration. (ii) Activity duration (hereafter activity), defined as time spent in any activity (walking, jumping, climbing etc.) and expressed as proportion on total duration of the test. Differences in activity levels may indicate a differential propensity to explore an unknown environment (Kelleher et al., 2018 and reference therein). (iii) Duration of time spent active on the arena floor (hereafter floor), defined as time spent active on the arena floor and expressed as the proportion on total duration of the test. The use of the horizontal (arena floor) rather than vertical surfaces may reflect a greater propensity to move towards new environments, especially for an arboreal species. (iv) Duration of time spent jumping (hereafter jumping) expressed as proportion on the total activity duration. Differences in jumping, the predominantly locomotory mode in a tree frog, could reflect a differential propensity to jump possibly suggesting a distinct locomotory mode used to explore a novel environment.

2.2.3.3 Shelter test

I performed the shelter test to measure boldness-shyness behavioural axis (Cote et al., 2010b). I used a rectangular opaque arena (length 40 cm x height 25 cm) video-surveyed by the same camera as in the previous test (section 3.2.3.2). I gently transferred each individual from its home cage onto a cylindric dark shelter (diameter 6 cm) in the arena periphery. The dark shelter had a circular opening (diameter 2 cm) facing towards the centre of the arena and covered by a cap allowing an acclimation period of 6 minutes. After the acclimation period, I opened the shelter. The test ended when the frog left the shelter with a cut-off time of 10 minutes. After that, I immediately transferred back each individual to its home cage and I cleaned the arena as descripted above (section 3.2.3.2). I tested individuals randomly respect to the population of origin on the same day between 9:00 and 14:00. Later I analysed the individual behaviour by using the software Boris 5.1.3. I recorded the latency to exit from the shelter (hereafter boldness). A shorter latency is assumed to reflect greater inclination to take risks being easily exposed to predators in an open space, indicating a bold personality (Brown et al., 2007; Cote et al., 2010b; Beckmann and Biro, 2013).

2.2.4 Data Analysis

All statistical analyses were run using R software version 3.5.3 (R Core Team 2019). First, I performed Generalized Linear Models (glm in package 'nlme') to test whether spontaneous activity in a familiar environment differed between islands. I fitted four different models entering each variable singly (inactivity and inactivity on green) and island as fixed factor. I analysed data from Sardinia *vs*. Corsica separately from that of Corsica *vs.* Elba island. A Gaussian distribution with identity link function was used for inactivity, while a Gamma distribution and a log link function was used for the inactivity on green. Preliminary models showed that entering individual body mass as covariate (Kelleher et al., 2017), both with and

without interaction with island, did not improve the fitting of the model (i.e., the Akaike Information Criterion value was not reduced beyond 2), thus it was no longer considered.

When I found significant differences between the islands, I used the same approach of before to test whether the spontaneous activity traits differed even among populations from Sardinia *vs.* Corsica and Corsica *vs.* Elba island, separately.

I ran Generalized Linear Mixed effect (GLMM)-based repeatability models (glmer in package 'lme4') to test the within-individual repeatability of all behavioural traits (Nakagawa and Schielzeth, 2010). Gamma distribution with a log link function was used. The observation-level variance was obtained by using the trigamma function (Nakagawa et al., 2017). I entered each behavioural trait as a dependent variable and individual as random factor. Data from Sardinia *vs*. Corsica were analysed separately from that of Corsica *vs.* Elba island. Behavioural variables were considered as personality traits and used for the further analysis when their repeatability value was $R > 0.2$, as reported by previous studies documenting animal personality in other anurans (Brodin et al., 2013; Kelleher et al., 2017; Kelleher et al., 2018 and references therein). Preliminary models showed that the adjusted repeatability calculated by entering population as fixed factor did not improve the fitting of the models (i.e., the Akaike Information Criterion value was not reduced beyond 2), thus it was no longer considered.

I performed Linear Models (lm in package 'lme4') to evaluate differences in personality traits between islands. I used Linear Models (LMs) because they improve the fitting of the models (i.e., the Akaike Information Criterion value was reduced beyond 2), in respect to the GLMs.

Dependent variables were $\log_{10}$ transformed to meet the assumption of residuals normality. I fit five different models entering each variable singly and island as fixed factor. Data from Sardinia *vs*. Corsica were analysed separately from that of Corsica *vs.* Elba island. Preliminary models showed that entering body mass as covariate (Kelleher et al., 2017), both with and without interaction with island, did not improve the fitting of the model (i.e., the Akaike Information Criterion value was not reduced beyond 2), thus it was no longer considered. I

used the same approach reported above to check whether the same traits were even different among populations from Sardinia *vs.* Corsica and Corsica *vs.* Elba island, separately.

2.3 Results

2.3.1 Spontaneous behaviour

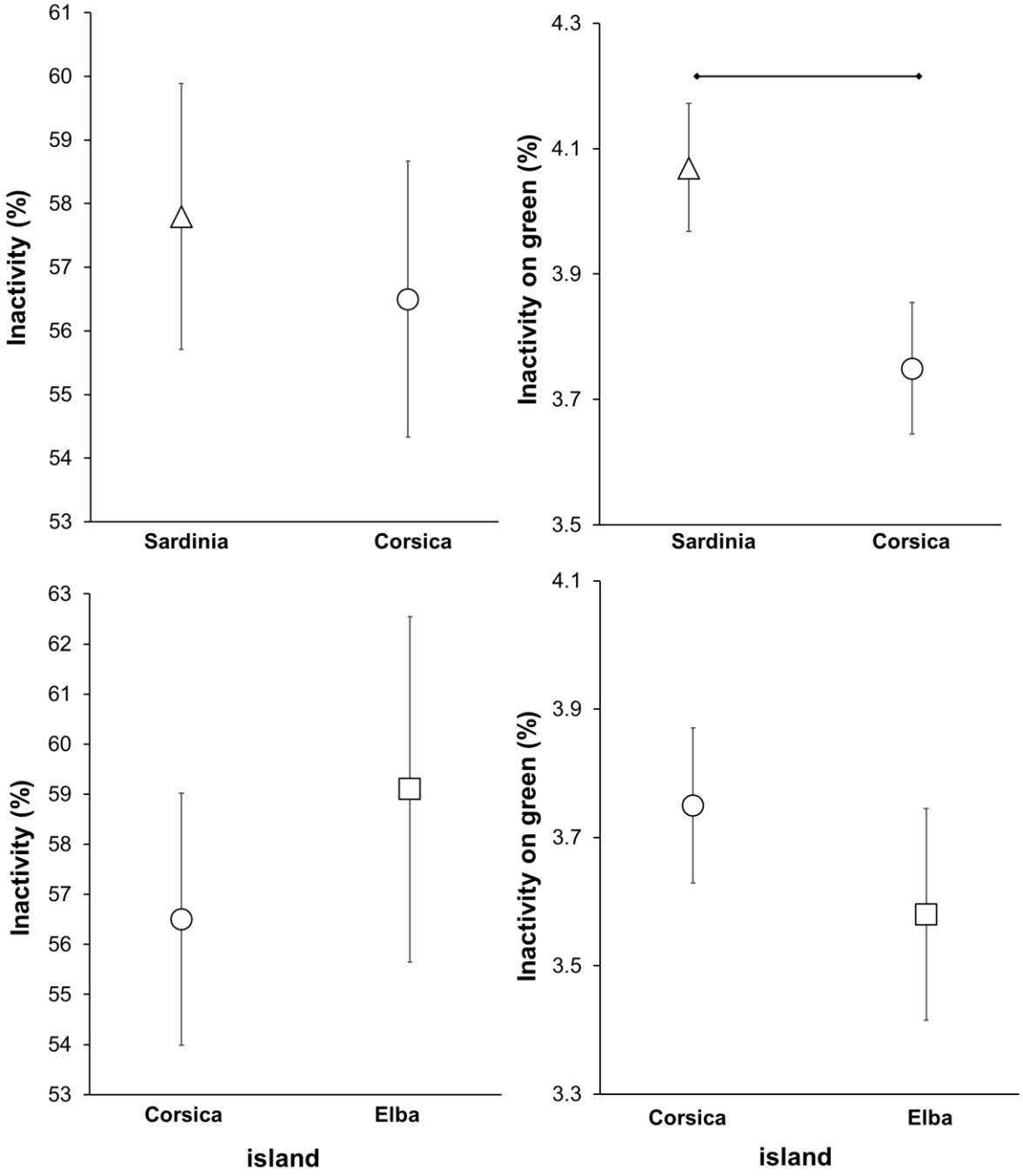
I found that Sardinia and Corsica frogs did not differ in the inactivity, while significant differences were found in the inactivity on green (Table 2). Sardinia frogs prefer a green substrate as compared to those from Corsica (S=4.07 ± 0.102; C=3.75 ± 0.105, p-value=0.028). The Estimated Marginal Means (EMMs) from Generalized Linear Models (GLMs) are provided in Figure 2.

I did not find any differences between Corsica and Elba island in both the spontaneous activity traits (Table 2 and Figure 2).

Table 2 Coefficient estimates (± SE) of GLMs with spontaneous activity traits as dependent variables and island as fixed factor. Gaussian distribution with identity link function was used for inactivity while, Gamma distribution with log link function was used for inactivity on green. Data sets of Sardinia-Corsica and Corsica-Elba have been analysed separately. S=Sardinia; C=Corsica; E=Elba island. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	P
inactivity	S	C	-1.297	3.016	-0.43	0.668
	C	E	2.545	4.273	0.596	0.554
inactivity on green	S	C	0.327	0.146	2.237	**0.028**
	C	E	-0.165	0.204	-0.807	0.423

Figure 2 EMMs and standard errors from GLMs for spontaneous activity traits of Sardinia-Corsica (top) and Corsica-Elba (bottom). S=Sardinia (triangle), C=Corsica (circle) and E=Elba island (square). Inactivity=percentage of inactivity events; Inactivity on green=percentage of inactivity events on a green substrate. Significant post-hoc (Tukey) contrasts are indicated with a bar.



At population scale, the inactivity on green differed between south Sardinia (SS) and north Corsica (NC), between north Sardinia (NS) and north Corsica and within Corsica island between its south (SC) and north population (Table 3). Moreover, I found a decreasing pattern

starting from south Sardinia up to north Corsica, (SS=3.97 ± 0.14; NS=4.17 ± 0.14; SC=3.94 ± 0.14; NC=3.46 ± 0.15), with south Sardinia and north Sardinia having almost comparable values (Figure 3).

I did not find differences among Corsica and Elba island populations (Table 4 and Figure 3).

Table 3 Coefficient estimates (± SE) of GLMs with spontaneous activity traits as dependent variable and Sardinia-Corsica populations as fixed factors. Gaussian distribution with identity link function was used for inactivity while, Gamma distribution with log link function was used for inactivity on green. SS=South Sardinia; NS=North Sardinia; SC=South Corsica; NC=North Corsica. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	P
inactivity	SS	NS	-3.047	4.225	-0.721	0.473
	SS	SC	-3.393	4.225	-0.803	0.424
	SS	NC	-2.157	4.389	-0.492	0.624
	SC	NC	1.236	4.389	0.282	0.779
	SC	NS	0.346	4.225	0.082	0.935
	NC	NS	-0.889	4.389	-0.203	0.840
inactivity on green	SS	NS	0.1939	0.194	0.978	0.331
	SS	SC	-0.033	0.198	-0.169	0.866
	SS	NC	-0.507	0.206	-2.463	**0.016**
	SC	NC	-0.474	0.206	-2.300	**0.024**
	SC	NS	0.227	0.198	1.147	0.255
	NC	NS	0.701	0.206	3.404	**0.001**

Figure 3 EMMs and standard errors from GLMs for spontaneous activity traits among Sardinia-Corsica (on top) and Corsica-Elba (on bottom) populations. SS=South Sardinia (white triangle); NS=North Sardinia (black triangle); SC=South Corsica (white circle); NC= North Corsica (black circle); EL=Elba (white square). Significant post-hoc (Tukey) contrasts are indicated with a bar.

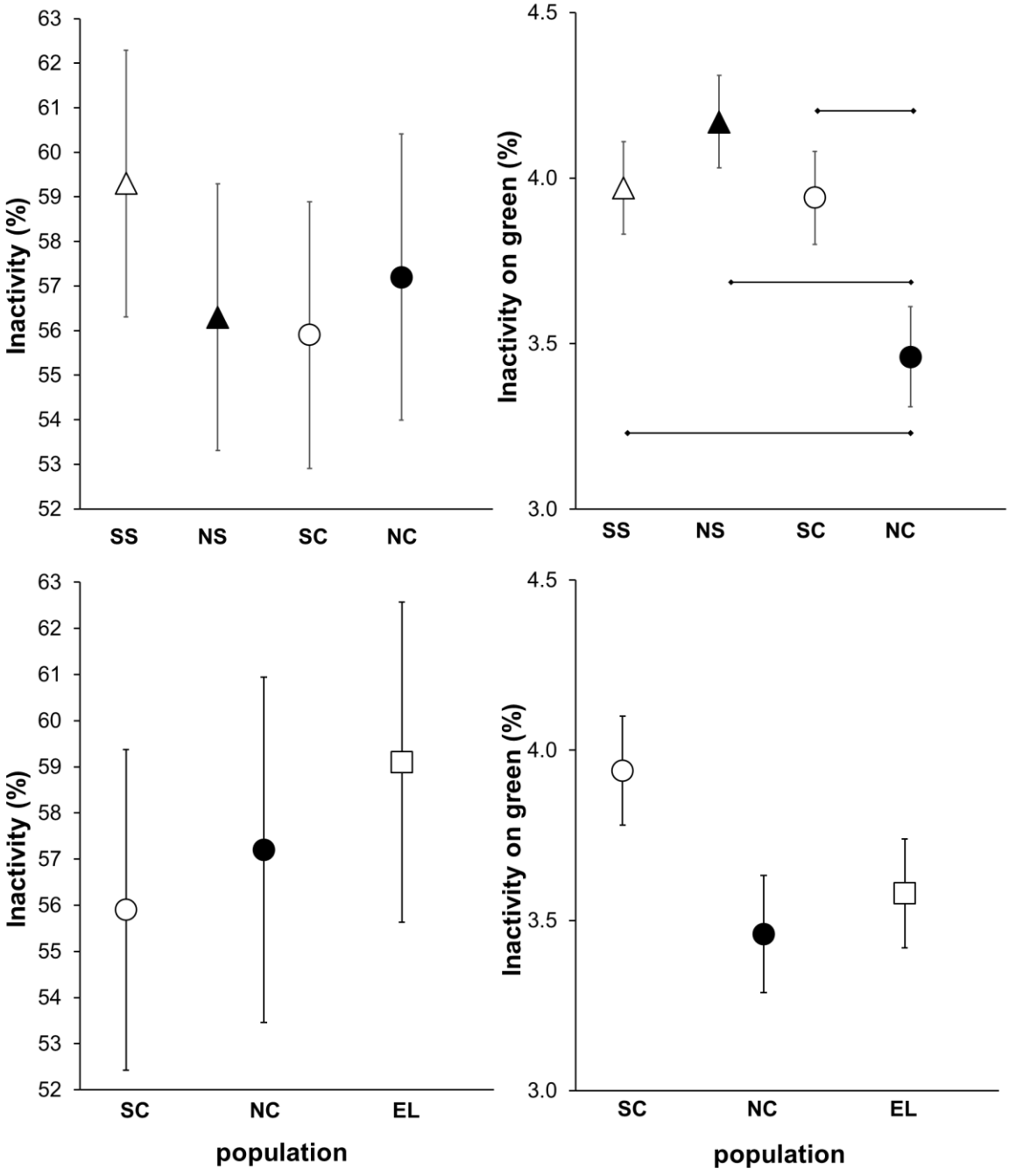


Table 4 Coefficient estimates (± SE) of GLMs with spontaneous activity traits as dependent variable and Corsica-Elba populations as fixed factors. Gaussian distribution with identity link function was used for inactivity while, Gamma distribution with log link function was used for inactivity on green. SC=South Corsica; NC=North Corsica; EL=Elba. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	P
inactivity	NC	SC	-1.236	5.103	-0.242	0.809
	NC	EL	1.882	5.103	0.369	0.714
	SC	EL	3.117	4.192	0.635	0.528
inactivity on green	NC	SC	-0.012	0.006	-1.900	0.062
	NC	EL	-0.003	0.007	-0.494	0.623
	SC	EL	0.008	0.005	1.540	0.129

2.3.2 Novel Environment test

All behavioural traits were significantly repeatable over time for both the datasets analysed, Sardinia *vs.* Corsica and Corsica *vs.* Elba island. The repeatability coefficient with its observation-level variance of the measured behavioural traits are reported in Table 5 for Sardinia *vs.* Corsica and in Table 6 for Corsica *vs.* Elba island.

Table 5 Summary of GLMM-based repeatability estimates for Sardinia *vs*. Corsica. Behavioural traits were dependent variables and individual was a random factor. Gamma distribution with log link function was used. The observation-level variance was obtained by using the trigamma function.

Variable	Estimate	Observation-level variance
latency	0.44	0.4106
activity	0.26	0.3985
floor	0.48	1.4630
jumping	0.34	0.0244

Table 6 Summary of GLMM-based repeatability estimates for Corsica *vs*. Elba island. Behavioural traits were dependent variables and individual was a random factor. Gamma distribution with log link function was used. The observation-level variance was obtained by using the trigamma function.

Variable	Estimate	Observation-level variance
latency	0.38	0.5346
activity	0.24	0.5043
floor	0.48	1.6085
jumping	0.30	0.0281

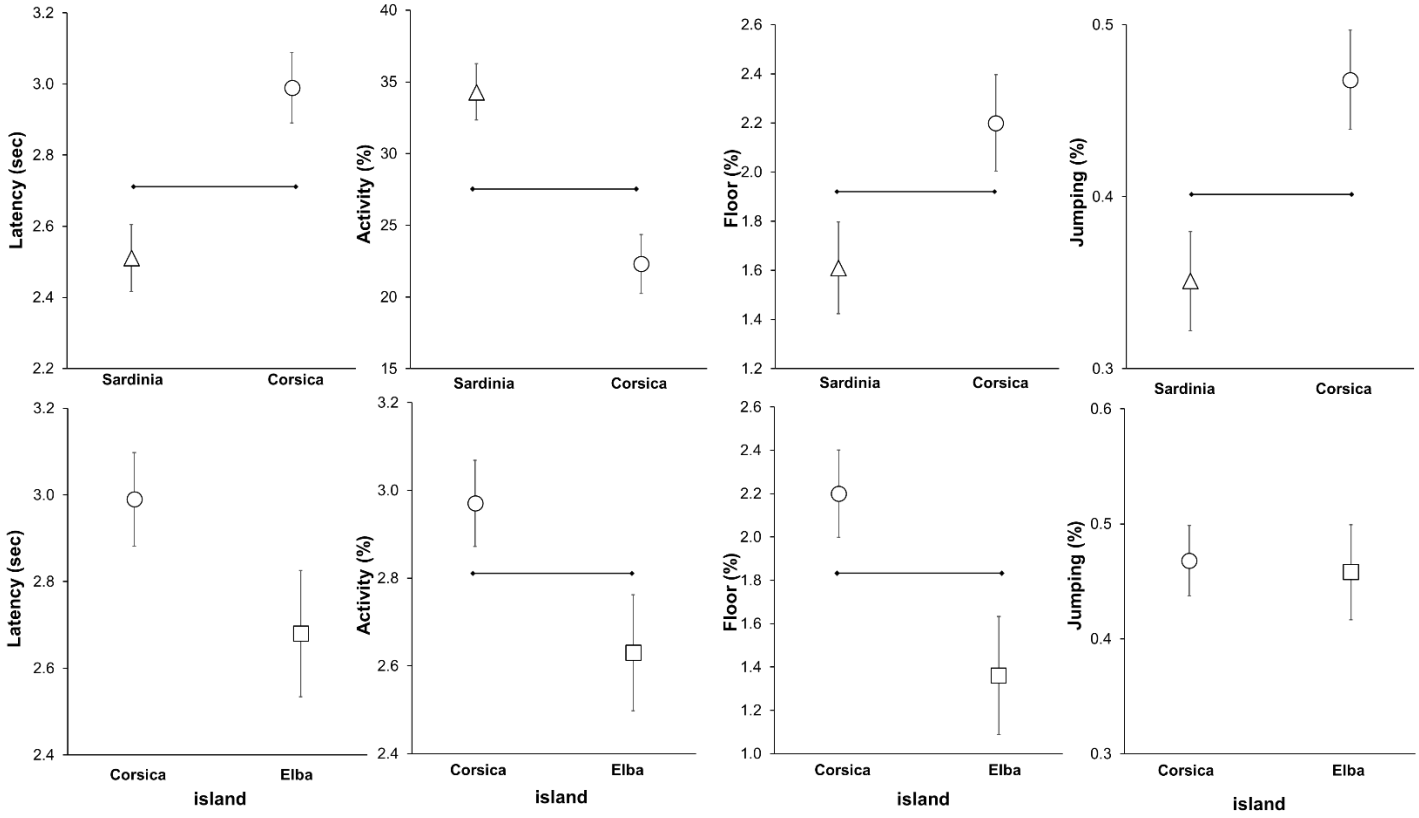
I found significant differences in all personality traits between Sardinia and Corsica (Table 7). Tree frogs from Sardinia had a shorter latency and were more active than those from Corsica. In fact, the EMMs from Linear Models (LMs) showed that latency time was higher in Corsica than in Sardinia (S=2.51 ± 0.094; C=2.99 ± 0.099, p-value=0.0006) indicating a more prudent personality in Corsica. Similarly, tree frog from Corsica were significantly less active than those from Sardinia (S=34.3 ± 1.96; C=22.3 ± 2.06, p-value<0.0001). On the contrary, time spent active on the arena floor (S=1.61± 0.187; C=2.2 ± 0.196, p-value=0.0323) as well as time spent jumping (S=0.35 ± 0.029; C=0.47 ± 0.030, p-value=0.0066) were significantly higher in Corsica than in Sardinia. The EMMs values for personality differences between Sardinia-Corsica are given in Figure 4.

In the comparison between Corsica and Elba island, I found significant differences in activity and in time spent active on the arena floor while, latency and jumping did not differ (Table 7). Specifically, tree frogs from Elba island were less active than those from Corsica (C=2.97± 0.098; E=2.63 ± 0.132, p-value=0.0434) and spent a significant smaller amount of time on the arena floor (C=2.2 ± 0.202; E=1.36 ± 0.273, p-value=0.0162). The EMMs values for personality differences between Corsica-Elba are provided in Figure 4.

Table 7 Coefficient estimates (± SE) of LMs with personality traits as dependent variable and island as fixed factor. Data sets of Sardinia-Corsica and Corsica-Elba have been analysed separately. S=Sardinia; C=Corsica; E=Elba island. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	P
latency	S	C	0.486	0.136	3.558	**0.0006**
	C	E	-0.318	0.181	-1.752	0.0849
activity	S	C	-12.050	2.842	-4.24	**<0.0001**
	C	E	-0.340	0.165	-2.063	**0.0434**
floor	S	C	0.588	0.270	2.177	**0.0323**
	C	E	-0.839	0.339	-2.473	**0.0162**
jumping	S	C	0.116	0.042	2.788	**0.0066**
	C	E	-0.009	0.051	-0.188	0.8521

Figure 4 EMMs and standard errors from LMs for personality traits of Sardinia-Corsica (on top) and Corsica-Elba (on bottom). S=Sardinia (triangle), C=Corsica (circle) and E=Elba island (square). Latency= latency (sec); activity= time spent active (expressed as percentage on total test duration); floor= time spent on the arena floor (expressed as percentage on total test duration); jumping= time spent in jumping (expressed as percentage on total activity). Significant post-hoc (Tukey) contrasts are indicated with a bar.



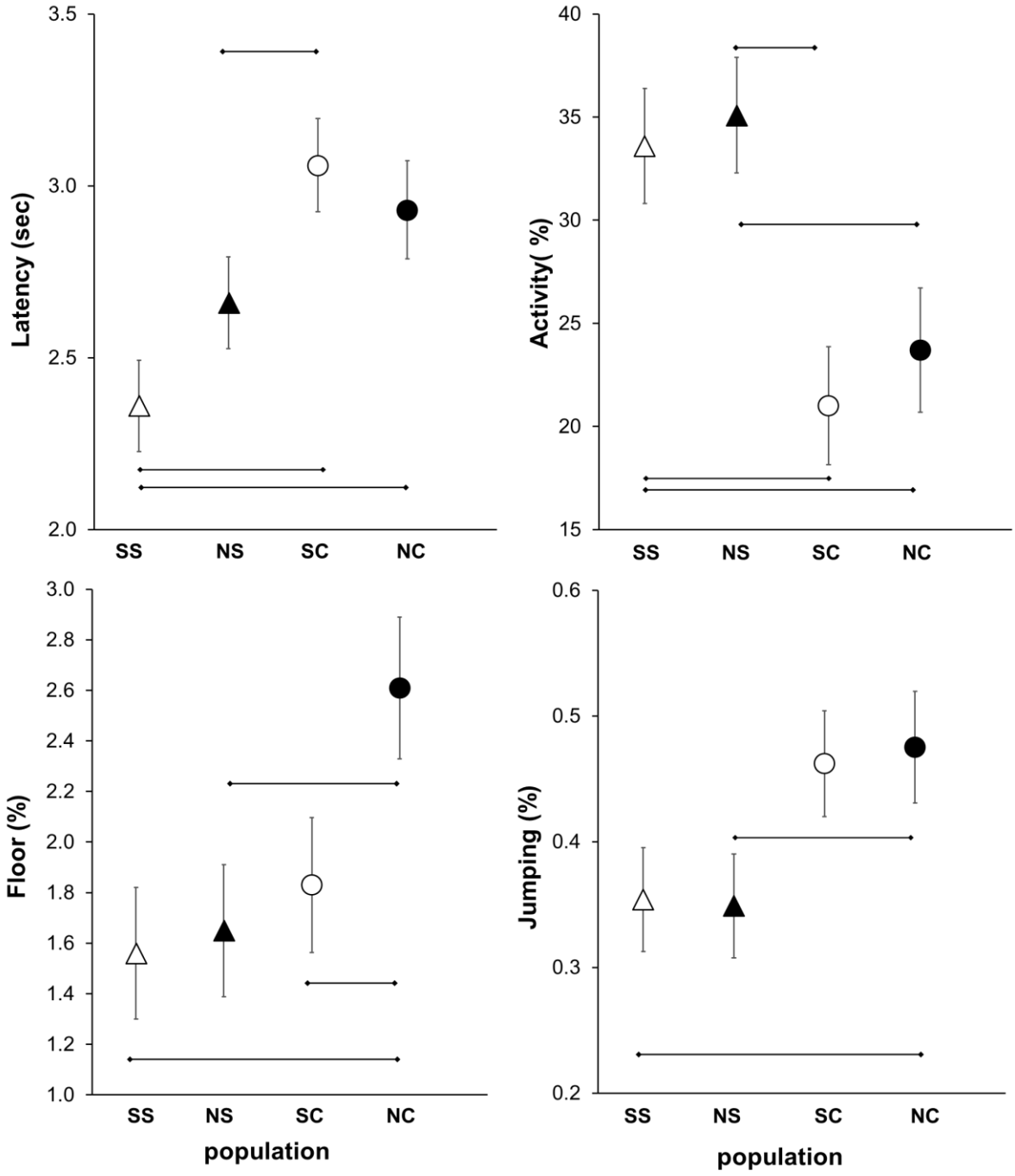
At population scale, all personality traits differed significantly ($p<0.05$) between south Sardinia (SS) and north Corsica (NC). I also found marked differences between the north Sardinia (NS) and south Corsica (SC), in the activity (NS=35.1 ± 2.8; SC=21 ± 2.86, p-value=0.0006) and latency (NS=2.66 ± 0.133; SC=3.06 ± 0.136, p-value=0.0376). I found a strong differentiation between the south Sardinia and south Corsica, at least in the activity (SS=33.6 ± 2.8; SC=21 ± 2.86, p-value=0.0023) and latency (SS=2.36 ± 0.133; SC=3.06 ± 0.136, p-value=0.0004). Substantial differences were also found between north Sardinia and north Corsica at least in activity (NS=35.1 ± 2.08; NC=23.7 ± 3.01, p-value=0.0072), jumping (NS=0.35 ± 0.041; NC= 0.47 ± 0.044, p-value=0.0406) and floor (NS=1.65 ± 0.261; NC= 2.61 ± 0.28, p-value=0.0147). The latter (floor) is the only personality traits that differed within Corsica, between its south and north population (SC=1.83 ± 0.267; NC=2.61 ± 0.28, p-value=0.0467). I did not findany personality differences within Sardinia. Interestingly, the time spent on the arena floor showed increasing EMMs starting from south Sardinia up to north Corsica (SS=1.56 ± 0.261; NS=1.65 ± 0.261; SC=1.83 ± 0.267; NC=2.61 ± 0.28). A similar pattern was found for latency (SS=2.36 ± 0.133; NS=2.66 ± 0.13; SC=3.06 ± 0.136; NC=2.93 ± 0.143).

A summary of the parameter estimates used to investigate population differences in personality traits is reported in Table 8 while the EMMs values for personality differences among Sardinia-Corsica populations are provided in Figure 5.

Table 8 Coefficient estimates (± SE) of LMs with personality traits as dependent variable and Sardinia-Corsica populations as fixed factors. SS=South Sardinia; NS=North Sardinia; SC=South Corsica; NC=North Corsica. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	Ps(>|t|)
latency	SS	NS	0.294	0.187	1.569	0.1206
	SS	SC	0.695	0.189	3.664	**0.0004**
	SS	NC	0.565	0.195	2.899	**0.0048**
	SC	NC	-0.130	0.197	-0.663	0.5093
	SC	NS	-0.401	0.189	-2.114	**0.0376**
	NC	NS	-0.270	0.195	-1.389	0.1687
activity	SS	NS	1.488	3.957	0.376	0.7079
	SS	SC	-12.623	4.004	-3.153	**0.0023**
	SS	NC	-9.852	4.110	-2.397	**0.0189**
	SC	NC	2.771	4.155	0.667	0.5068
	SC	NS	14.111	4.004	3.524	**0.0007**
	NC	NS	11.340	4.110	2.759	**0.0072**
floor	SS	NS	0.089	0.368	0.243	0.8086
	SS	SC	0.262	0.373	0.703	0.4839
	SS	NC	0.484	0.383	2.728	**0.0078**
	SC	NC	0.782	0.387	2.021	**0.0467**
	SC	NS	-0.173	0.373	-0.463	0.6444
	NC	NS	-0.954	0.383	-2.494	**0.0147**
jumping	SS	NS	-0.006	0.058	-0.097	0.9232
	SS	SC	0.107	0.059	1.820	0.0725
	SS	NC	0.120	0.061	1.988	**0.0502**
	SC	NC	0.0130	0.061	0.213	0.8320
	SC	NS	-0.113	0.059	-1.915	0.0590
	NC	NS	-0.126	0.061	-2.081	**0.0406**

Figure 5 EMMs and standard errors from LMs for personality traits among Sardinia-Corsica populations. SS=South Sardinia (white triangle); NS=North Sardinia (black triangle); SC=South Corsica (white circle); NC=North Corsica (black circle). Significant post-hoc (Tukey) contrasts are indicated with a bar.



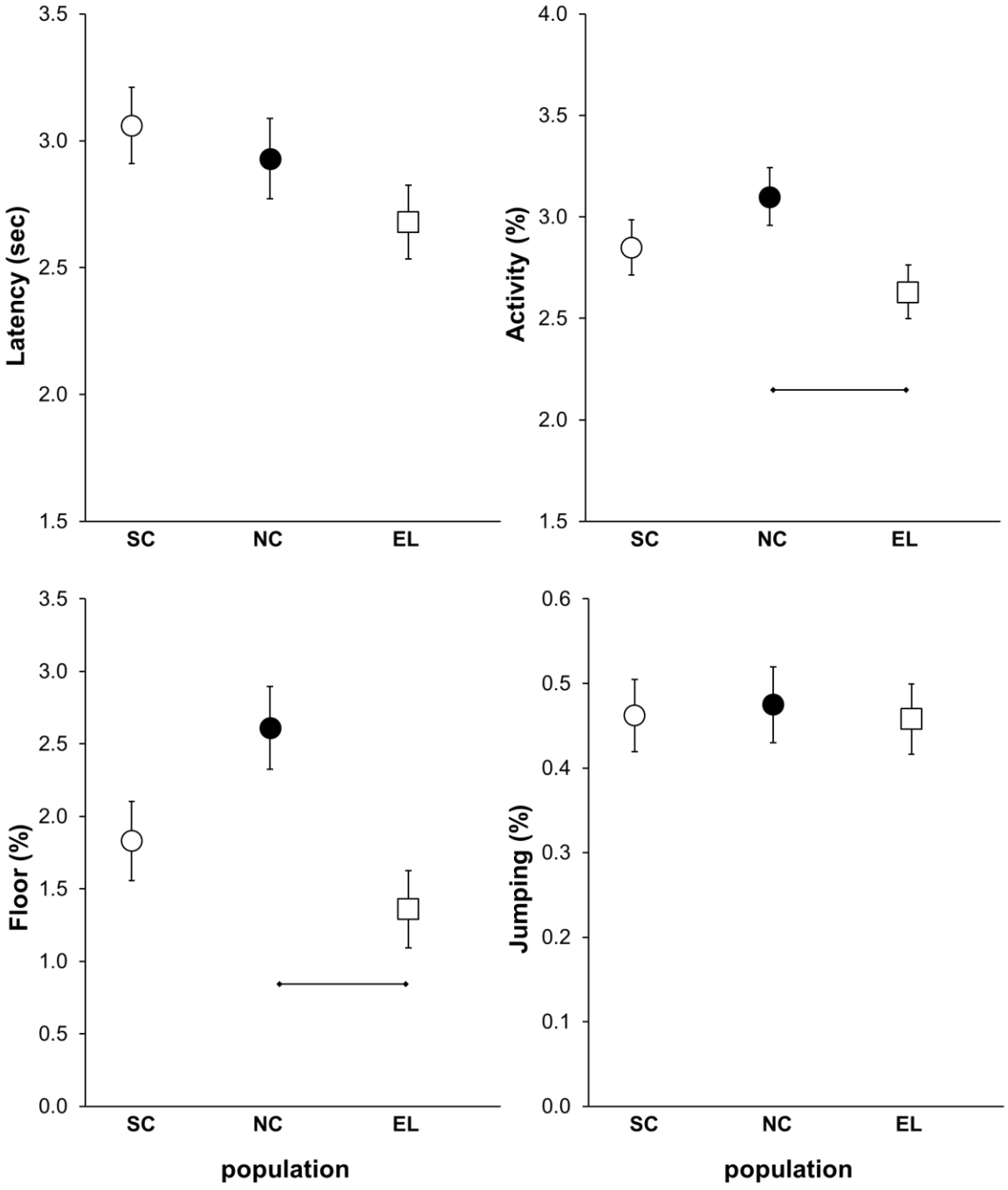
I found, among Corsica and Elba island populations, significant differences between NC and EL in the activity and time spent on the arena floor while, I did not find differences between the SC and EL. I found the same EMMs pattern in the activity and the time spent on the arena

floor , with growing values within Corsica, from south up to north, that sharply decreased in the Elba island (activity: CS=2.85 ± 0.135; NC=3.1 ± 0.142;EL=2.63 ± 0.132 floor: CS=1.83 ± 0.272; NC=2.61 ± 0.286; EL=1.36 ± 0.266). A summary of the parameter estimates used to investigate population differences in personality traits is reported in Table 9 while the EMMs values are given in Figure 6.

Table 9 Coefficient estimates (± SE) of LMs with personality traits as dependent variable and Corsica-Elba populations as fixed factors. SC=South Corsica; NC=North Corsica; EL=Elba. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	Ps(>|t|)
latency	NC	SC	0.130	0.218	0.600	0.551
	NC	EL	-0.249	0.215	-1.159	0.251
	SC	EL	-0.379	0.210	-1.812	0.075
activity	NC	SC	-0.246	0.196	-1.255	0.214
	NC	EL	-0.469	0.194	-2.423	**0.018**
	SC	EL	-0.223	0.189	-1.185	0.241
floor	NC	SC	-0.782	0.395	-1.977	0.053
	NC	EL	-1.250	0.391	-3.196	**0.002**
	SC	EL	-0.468	0.381	-1.228	0.224
jumping	NC	SC	-0.013	0.062	-0.211	0.834
	NC	EL	-0.016	0.061	-0.270	0.788
	SC	EL	-0.003	0.059	-0.058	0.954

Figure 6 EMMs and standard errors from LMs for personality traits among Corsica-Elba populations. SC=South Corsica (white circle); NC=North Corsica (black circle); EL=Elba island (white square). Significant post-hoc (Tukey) contrasts are indicated with a bar.



2.3.3 Shelter test

Boldness was significantly repeatable over time for both the datasets analysed, Sardinia *vs.* Corsica and Corsica *vs.* Elba island. The repeatability coefficient with its observation-level variance of boldness is reported in Table 10 for both the datasets analysed.

Table 10 Summary of GLMM-based repeatability estimates for Sardinia *vs*. Corsica and Corsica *vs.* Elba. Boldness was dependent variable and individual was a random factor. Gamma distribution with log link function was used. The observation-level variance was obtained by using the trigamma function.

Dataset	Estimate	Observation-level variance
Sardinia-Corsica	0.59	0.3454
Corsica-Elba	0.67	0.2587

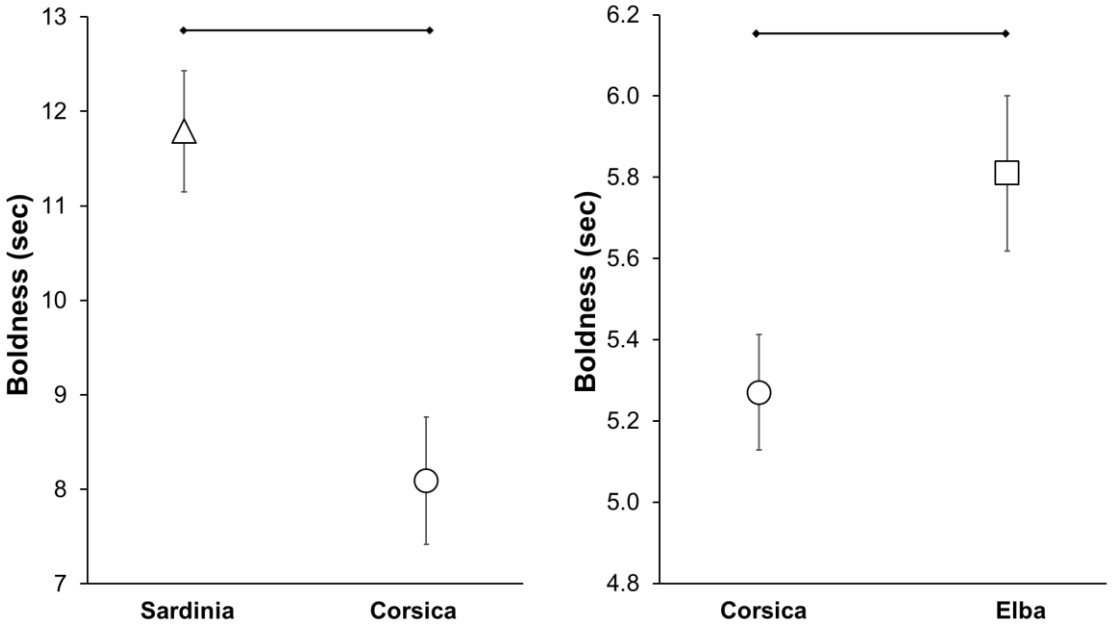
Boldness differs significantly between Sardinia and Corsica. Specifically, tree frogs from Corsica were less bold than those from Sardinia (S=11.79 ± 0.642; C=8.09 ± 0.673).

I also found marked differences between Corsica and Elba island. Tree frogs from Elba island were significantly bolder than those inhabiting Corsica (C=5.27 ± 0.142; E=5.81 ± 0.191). A summary of the parameter estimates used to investigate boldness differences among islands is reported in Table 11. The EMMs values for boldness differences between Sardinia and Corsica and between Corsica and Elba island are reported in Figure 7.

Table 11 Coefficient estimates (± SE) of LMs with boldness as dependent variable and island as fixed factor. Data sets of Sardinia-Corsica and Corsica-Elba have been analysed separately. S=Sardinia; C=Corsica; E=Elba island. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	P
boldness	S	C	-3.701	0.929	-3.981	**<0.0001**
	C	E	-0.5372	0.238	-2.254	**0.0279**

Figure 7 EMMs and standard errors from LMs for boldness of Sardinia-Corsica (on left) and Corsica-Elba (on right). S=Sardinia (triangle), C=Corsica (circle) and E=Elba island (square). Boldness= inverse of the time to exit from a shelter. Significant post-hoc (Tukey) contrasts are indicated with a bar.



I found, among Sardinia and Corsica populations, marked differences in boldness between north Sardinia (NS) and south Corsica (SC) (NS=11.6 ± 0.918; SC=7.91 ± 0.918) and between south Sardinia (SS) and south Corsica (SS=11.97 ± 0.918; SC=7.91 ± 0.918). I also found substantial differences between north Corsica (NC) and south Sardinia (NC=8.31± 1.014; SS=11.97 ± 0.918) and between north Sardinia and north Corsica (NS=11.6 ± 0.918; NC=8.31± 1.014). A summary of the parameter estimates used to investigate population differences in boldness is reported in Table 12 for Sardinia *vs.* Corsica.

Table 12 Coefficient estimates (± SE) of LMs with boldness as dependent variable and Sardinia-Corsica populations as fixed factors. SS=South Sardinia; NS=North Sardinia; SC=South Corsica; NC=North Corsica. Significant contrasts are shown in bold.

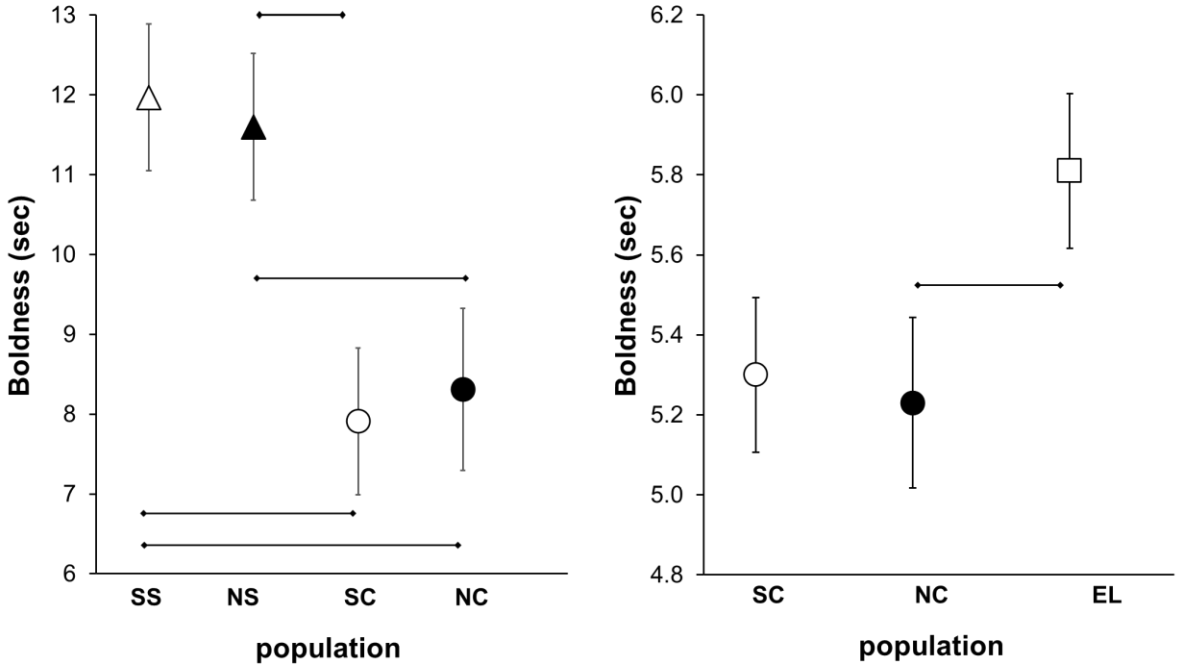
Dependent variable	Reference Level	Level	Coefficient	SE	t value	Ps(>|t|)
boldness	SS	NS	-0.365	1.297	0.282	0.7790
	SS	SC	4.064	1.298	3.132	**0.0024**
	SS	NC	3.663	1.368	2.678	**0.0089**
	SC	NC	-0.401	1.367	-0.293	0.7703
	SC	NS	-3.699	1.298	-2.850	**0.0055**
	NC	NS	-3.298	1.368	-2.411	**0.0181**

I found, among Corsica and Elba island populations, significant differences in boldness between north Corsica and Elba, while I did not find any differences between the south Corsica and Elba (Table 13). The EMMs values for boldness differences between Sardinia and Corsica and between Corsica and Elba island are reported in Figure 8.

Table 13 Coefficient estimates (± SE) of LMs with personality traits as dependent variable and Corsica-Elba populations as fixed factors. SC=South Corsica; NC=North Corsica; EL=Elba. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	Ps(>|t|)
boldness	NC	SC	-0.071	0.288	-0.246	0.806
	NC	EL	-0.576	0.287	-2.003	**0.049**
	SC	EL	-0.505	0.273	-1.852	0.069

Figure 8 EMMs and standard errors from LMs for boldness among Sardinia-Corsica (on left) and Corsica-Elba (on right) populations. SC=South Corsica (white circle); NC=North Corsica (black circle); EL=Elba island (white square). Boldness=inverse of the time to exit from a shelter. Significant post-hoc (Tukey) contrasts are indicated with a bar.



2.4 Discussion

In this study, I investigated whether historical biogeographic processes have produced a differentiation in personality traits among three islands (Sardinia, Corsica and Elba) distributed along the inferred route of the *H. sarda* past expansion. To test a personality differentiation as a consequence of a Pleistocene range expansion, I compared Sardinia with Corsica, while the effects of a past island colonization trough jump dispersal were investigated by comparing the same traits between Corsica and Elba. Below, I discussed the results from these two comparisons (Sardinia *vs.* Corsica and Corsica *v*s. Elba) separately.

2.4.1 Historical biogeography of personality: range expansion through spatial diffusion

Here I provide evidence of a significant differentiation in all the studied personality traits along the Sardinia-Corsica route of the *H. sarda* past expansion. Tree frogs from Corsica were significantly shyer, less active and with longer decision-making time (latency) than their ancestors (Sardinia). On the other hand, tree frogs from Corsica preferred a jump locomotory mode compared to those from Sardinia. Moreover, tree frogs from north Corsica showed a significantly higher use of horizontal surfaces than those from Sardinia.

The absence of remarkable differences in the levels of genetic variability coupled with the lack of genetic structure between Sardinia and Corsica populations (Bisconti et al., 2011b) might suggest that the past range expansion has been promoted by a random sample of individuals. However, this scenario would not explain a geographic structure of the studied traits that appear assorted along the past south-north expansion axis. All personality traits (except for time spent on the arena floor as well as jumping) showed substantial differences between south Sardinia, the putative glacial refuge, and south Corsica, which would be the first colonization area in Corsica. Interestingly, the same traits differed also between northern Sardinia and southern Corsica, where there was the wide and suitable land-bridge area that connected the two islands

during the expansion phase (Thiede, 1978). These patterns suggest that individuals that successfully arrived in Corsica crossing the land bridge, may have been a non-random subset of behavioural phenotypes of the ancestral population (Sardinia). This interpretation is in line with several theoretical hypotheses and empirical studies of personality dependent dispersal (Fraser et al., 2001; Dingemanse et al., 2003; Cote and Clobert, 2007; Cote et al., 2010a; Canestrelli et al., 2016a). However, my results are in contrast with the expected combination of personality traits in a non-random expansion process. Indeed, dispersers at the range-edge have frequently been found bolder and more exploratory than those living in long established areas (Pintor et al., 2008; Liebl and Martin, 2012; Atwell et al., 2012; Myles-Gonzalez et al., 2015; Gruber et al., 2017a). Instead, we found that dispersers at the range-edge (Corsica) are shyer and characterized by a prudent exploratory strategy compared to those living in the ancestral areas (Sardinia). At first instance, our results would seem counterintuitive but considering the species' ecology, it is plausible that those individuals that successfully arrived in Corsica could be advantaged by evolving a shy personality. A shy personality, revealed also by a long decision-making time (latency), could allow a more careful assessment of the surrounding environment with a significant reduction of risks, ultimately increasing the chance of survival. Similarly, the low activity rate could have given significant advantages to dispersers both in term of risks reduction and energy optimization in an environment, where both the abundance and the availability of resources were unknown. Furthermore, the use of the horizontal surfaces (reflected by time spent on the arena floor) rather than vertical, might have favoured those individuals who expressed this propensity because relying more on horizontal than vertical movement may promote the expansion, at least through non-wooded coastal plains. Interestingly, this is the only trait that showed significant individual variation within Corsica with increasing values from south up to north. Finally, the higher propensity to jump might have favoured a cost-benefit optimization for dispersers. Although a jumping locomotory mode is energetically costly, it may allow covering long distances with a single movement

interspersed with pauses to recover energy. Moreover, these pauses could have given the opportunity to dispersers to hide from predators and, more generally, to assess carefully the surrounding environment before to explore again.

The contrast of my results with those expected from literature (Pintor et al., 2008; Atwell et al., 2012; Liebl and Martin, 2012; Myles-Gonzalez et al., 2015; Gruber et al., 2017a) could be related to the temporal dimension of the investigated process. To my knowledge, this is the first study that provides evidence of persistent differentiation in personality traits as a consequence of a historical expansion. In contrast with previous studies, I did not collect dispersers on their route but instead, the descendants of those that successfully arrived and established in Corsica. The evolution of the observed differences in personality between the two islands should have occurred during the range expansion process or later.

2.4.2 Historical biogeography of personality: island colonization through jump dispersal

I provide evidence of a substantial differentiation in personality traits across populations that have long been isolated as a consequence of Elba island colonization through jump dispersal from Corsica (see chapter 2.2). Tree frogs from Elba island were significantly bolder than their ancestors (Corsica), which instead showed a more prudent personality. Moreover, tree frogs from Elba island spent a smaller amount of time on the arena floor and showed a markedly lower activity rate as compared to those from Corsica, which were more explorative. In short, Elba frogs were bolder but less exploratory than their ancestors (Corsica).

One plausible scenario that could explain the origin of such personality differentiation is a past founder event. This scenario is supported by the low genetic diversity of Elba frogs, as compared to the rest of the species' range, as well as by its geographic location at the northern edge of the species' range (Bisconti et al., 2011b). A past founder event could have contributed to fix such traits thus explaining their persistence. Moreover, the Elba island isolation may have precluded the recruitment of other alleles, thus preventing the evolution of boldness and

exploratory behaviour over time. Alternatively, the personality differences may have been maintained over time by the 'Island Syndrome', a mechanism of adaptive differentiation to the the ecological relaxation generally occurring on islands (e.g. rodents: Adler and Levins, 1994; birds: Blondel, 2000; reptiles: Novosolov et al., 2013). Several studies indicate that predation, typically reduced on islands (MacArthur and Wilson, 1967; Blumstein and Daniel, 2005; Cooper, Pyron and Garland, 2014), is one of the most important forces in shaping the phenotype including personality traits (Bell, 2005; Dingemanse et al., 2007; Bell and Sih, 2007). It could be that a relaxation of selective pressures such as predation may have caused a shift in the behavioural repertoire of the founding population leading to an increase in the expression of bold phenotypes, as well as a decrease in explorative phenotypes. Moreover, islands tend to have both lower abundance and diversity of arthropods, which are the main food source for many reptiles and amphibians (Janzen, 1973; Olesen and Valido, 2003). It remains possible that a bold personality could be beneficial in an environment with a limited access to resources (De Meester et al., 2018).

Another alternative hypothesis, in agreement with studies of personality-dependent dispersal (Fraser et al. 2001; Philips, 2006; Cote et al., 2011), could be that the founding population was characterized by a non-random subset of personalities (Dingemanse et al., 2003; Chapple et al., 2012; Sih et al., 2012; Canestrelli et al., 2016a; Gruber et al., 2017a). The founding population might represent a non-random subset of bold and 'good-explorer' phenotypes that could successfully colonize the Elba. The boldness may have been maintained over time by the absence of a counter selective force in a relaxed ecological context, while the exploratory abilities of the founding population could have been lost over time as a consequence of trade-offs with other traits determining fitness (Biro and Stamps, 2008; Smith and Blumstein, 2008; Wolf and Weissing, 2012). Hence, during colonization, it could be possible that boldness and exploration were coupled and that insularity effect over time decoupled these traits leading to a bold but non-explorative personality on Elba. Unfortunately, the lack of studies that have

investigated the variation in personality traits following a past island colonization prevents any comparison with our results. Brodin et al. (2013) tested the effect of isolation on boldness and exploration among island populations, suggesting a personality differentiation in relation to isolation. However, their studied populations were connected to mainland by ongoing gene flow, thus preventing any comparison with our findings.

CHAPTER 3 LOCOMOTORY PERFORMANCE TRAITS

3.1 Introduction

Dispersal ability relies, to large extent, upon the individual locomotory performance defined here as the individual locomotion (e.g. acceleration) capacity (Garland and Losos, 1994; Irschick and Garland, 2001; Le Galliard et al., 2004; Husak et al., 2006). Dispersal ability is a key factor for expansion outcome, as observed in several *taxa* (Cwynar and MacDonald, 1987; Phillips et al., 2006, 2008; Hoffmann and Weeks, 2007; Louppe et al., 2017) and it is therefore logical to assume that locomotory performance is a key component of this ability. Theoretical models suggest that in the course of an expansion individuals may be spatially sorted by their dispersal ability (Phillips, Brown and Shine, 2010; Shine et al., 2011). Empirical evidence showed how this process has driven a deterministic and often rapid evolution in the locomotory traits of expanding populations (Travis and Dytham, 2002; Phillips, Brown and Shine, 2010; Shine et al., 2011). Although a deterministic evolution in morphological dispersal-enhancing traits has been extensively documented (Cwynar and MacDonald, 1987; Hoffmann and Weeks, 2007; Phillips et al., 2006; Forsman et al., 2010; Chuang and Peterson, 2016; Hudson et al., 2016a), evidence for the evolution of locomotory performance is still scanty (but see Phillips 2008; Alford et al., 2009; Louppe et al., 2017). Highly dispersive phenotypes of clawed frog (*Xenopus laevis*) were found at the invasion front in France. Those phenotypes showed a greater endurance, measured as the total distance covered and time spent moving until exhaustion, than those living in long-been invaded areas (Louppe et al., 2017). Another good example of the deterministic evolution in locomotory performance traits is the successful invasion of cane toad (*Rhinella marina*) in Australia. Toads at the invasion front disperse more frequently, for longer times and with straighter paths even showing a higher endurance to the distance travelled prior to exhaustion (Phillips et al., 2008; Alford et al., 2009; Llewellyn et al., 2010), than those living

in long-been invaded areas. Although these studies provide evidence of a directional evolution of performance traits in contemporary expanding populations, whether or not these changes persist in animal populations remains substantially unknown. Both theoretical and empirical evidence suggest that, when the expansion stops, low dispersal phenotypes are recruited through a flow from the back (Canestrelli et al., 2016a) thus inverting the trend in the new established populations (Travis and Dytham, 2002; Brown et al., 2007; Phillips et al., 2008; Brown et al., 2014).

Here I investigated whether a past expansion event may have induced a differentiation in locomotory performance traits among three islands (Sardinia, Corsica and Elba) distributed along the inferred route of *Hyla sarda* past expansion (Bisconti et al., 2011b, chapter 2.2). My main hypothesis is that, if individual variation in locomotory performance is a key trait in determining the species dispersal ability during a range expansion, a spatial structure in performance traits might emerge along the range of a recently expanded population.

I used standardised tests to characterize two locomotory performance traits tightly related to dispersal ability in my species: the maximum jumping force at take-off and the maximum adhesion force. The jumping force at take-off was measured to characterize the propulsive phase of jump (e.g. Marsh and John-Alder, 1994; Nauwelaerts and Aerts, 2006 and reference therein), which is the predominant mode of locomotion in tree frogs. As the distance covered with jumps is a function of the propulsion force generated at take-off, the jumping force at take-off may be considered a crucial trait in determining the dispersal ability in a tree frog.

The maximum adhesion force was measured because it is a functional adaptation to the predominantly arboreal life style of tree frogs (Duellman and Trueb, 1994; Reilly and Jorgensen, 2011; Wells, 2010). Tree frogs indeed have specialised sub-digital pads (toe pads), which adhere via an area-based wet adhesive mechanism (Emerson and Diehl, 1980; Federle et al., 2006; Smith et al., 2006). This versatile anatomical specialization is intrinsically linked to jumping performance. In fact, toe pads are crucial for a safe landing in arboreal tree frogs, as

missing the target could have much severe consequences in term of energy consumption (Bijma et al., 2016). In milk frogs (*Trachycephalus resinifictrix)*, even a single toe pad is sticky enough to hold its body weight at landing (Bijma et al., 2016). Given the crucial role it has in the landing phase, the adhesiveness is expected to influence the species dispersal ability.

To test whether a locomotory differentiation was caused by a Pleistocene range expansion, I compared Sardinia with Corsica while, the outcomes of a past island colonization were investigated by comparing the same traits between Corsica and Elba island.

3.2 Materials and Methods

3.2.1 Locomotory performance tests

I collected and housed individuals following the same procedure reported above (see chapter 2.2.1). To quantify locomotory performance traits, I carried out two tests: (i) the jumping test; (ii) the adhesiveness test. I used a total of 130 individuals for both the jumping and the adhesiveness test. I carried out tests in two separated sessions with an interval of 30 days from each other. I have fasted all individuals for two days before tests to minimize any difference in body mass, due to a differential food intake, which could have influenced the measured locomotory performance traits. Moreover, I weighted each individual was before each test (scale Acculab model ATILON ATL-224-I). I carried out all tests in the housing room to avoid any change in humidity and temperature conditions that directly affect body temperature and, in turn, task performance (Duellman and Trueb 1994; Navas et al., 2008).

3.2.1.1 Jumping test

I conducted the jumping test to quantify the maximum jumping force at take-off (hereafter jumping force). I used a rectangular arena (length 100 cm × height 50 cm) filmed with a video camera (Panasonic model no. DMC-FZ300) placed laterally to the setup that recorded all tests at 100 frames per second. To measure the jumping force, I gently took each individual from its home cage and equipped with an accelerometry data logger (two Axy-4 units, Technosmart, Rome, 9.15 x 15 x 4 mm, 1 gr weight including battery,) set to record triaxial acceleration (0 - 4 g) at 100 Hz. I used a thin and soft metallic string to firmly deployed the logger on the back of frogs. I used one end of the string to create a loop around the animal's body (just above the pelvic girdle), while I firmly bonded the other end to the logger. Pilot preliminary tests showed that this was the best solution guarantying standardization and less stress to the animals by

preventing any limitation in their mobility, as well as any possible cutaneous damage. After that, I turned-on the logger and I induced each individual to jump through a slight stimulation in its caudal region (Mitchell and Bergmann, 2016). I collected five jumps for each individual. The whole procedure lasted about three minutes per individual and, after that, I immediately transferred it back in its home cage after removal of the logger. To remove any chemical cues or secretion left by previous individuals, I cleaned the arena with dechlorinated water. I tested individuals randomly respect to the population of origin on the same day between 9:00 and 13:00.

I later downloaded data from the logger onto a PC using custom-made software (AXY Manager 2). From the downloaded logger data, I extracted the dynamic body acceleration for each dimension (x, y, z) considering a running mean of 30 Hz (Wilson et al., 2006; Shepard et al., 2008). The x axis of the logger measured sway, the y axis measured surge, and the z axis measured heave (Halsey et al., 2008). After that, I summed these values to produce the vector sum of dynamic body acceleration [VeDBA = $\sqrt{(A_x^2 + A_y^2 + A_z^2)}$] by using Framework 4 software (version 2.5). For each individual I quantified five VeDBA values, one for each jump and the highest value of VeDBA was chosen to calculate the individual maximum jumping force at take-off was calculated [VeDBA x mass] and considered for the following analysis. I used high speed videos to remove imperfect jumps, considered as such when individuals jumped on the arena walls. Moreover, I used videos to validate the data extraction from the logger. The validation consisted in testing if the VeDBA values corresponded to the exact moment of jumping take-off, defined as the instant in which individuals had the hind legs fully extended and still adhered to the jump plane.

3.2.1.2 The adhesiveness test

I carried out the adhesiveness test to quantify the maximum adhesion force (hereafter adhesiveness), a functional adaptation to the predominantly arboreal life style of tree frogs (Duellman and Trueb, 1994).

I used a smooth plastic rotating wheel (diameter 20 cm x depth 20 cm) with a moderate and constant angular speed (3 revolution x minute). The wheel had a circular transparent cap to prevent the animals from escaping. I used the same video camera mentioned above, (chapter 4.2.1.1) placed frontal to the setup, at a distance of 20 cm from the wheel to record all tests. Before to measure the adhesion force, I gently took each individual from its home cage and I fully hydrated it by immersing it in dechlorinated water for 1 minutes in order to minimize any difference in the individual hydration status. After the hydration procedure was ended, I buffered each individual with cotton to remove the water in excess and, I inserted it into the wheel, which I immediately closed. Afterwards, I turned-on the wheel and I recorded five falls for each individual. I considered as falls only those in which the individual's head was oriented in the same direction of the wheel rotation. The whole procedure lasted about five minutes per individual and then I transferred back the frog in its home cage. To remove any chemical cue or secretion left by previous individuals, I cleaned the arena with dechlorinated water. I tested individuals randomly respect to the population of origin on the same day between 9:00 and 13:00.

I used the software Tracker (version 4.11.0) to extract for each individual, 5 angles of fall (radian), one for each fall and the biggest value was chosen for the subsequent analyses. I calculated the maximum adhesion force $[\cos(\alpha) \times \text{mass} \times g]$ following the standard protocol of Barnes et al. (2006).

3.2.2 Data analysis

All statistical analyses were run using R software version 3.5.3 (R Core Team 2019).

First, I performed Linear Models (LM) using the lme4 package in R (version 3.5.3; R Core Team 2019) to test whether the two locomotor performance traits, jumping force and adhesiveness, differed between islands. Dependent variables were $\log_{10}$ transformed to meet the assumption of residuals normality. I fitted two different models (one for each performance trait) entering island as fixed factor and checking for all possible contrasts. I analysed data from Sardinia *vs*. Corsica separately from that of Corsica *vs.* Elba.

I used the same approach as before to assess whether the same locomotory performance traits differed among populations from Sardinia *vs*. Corsica and Corsica *vs.* Elba, separately. I fitted four different models, two to test differences among populations of Sardinia *vs.* Corsica and two to compare Corsica populations with those from Elba. In each model, I entered each performance trait as dependent variable and population as a fixed factor, checking for all possible contrasts.

3.3 Results

I found significant differences in both the locomotory performance traits between Sardinia and Corsica (Table 1). Tree frogs from Corsica showed higher values in both the performance traits respect to their ancestor from Sardinia. In fact, the EMMs values of jumping, measured as maximum jumping force at take-off (N), were higher in Corsica than in Sardinia (S=2.3 ± 0.048; C=2.43 ± 0.047, p-value=0.044). Similarly, tree frogs from Corsica had values of adhesiveness, measured as the maximum adhesion force (N), significantly higher than those from Sardinia (S=30.6 ± 0.984; C=33.4 ± 0.984, p-value=0.05).

Table 1 Coefficient estimates (± SE) of LMs with locomotory performance traits as dependent variable and island as fixed factor. Data sets of Sardinia-Corsica and Corsica-Elba have been analysed separately. S=Sardinia, C=Corsica; E=Elba island. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	Ps(>|t|)
jumping force	S	C	0.1372	0.0673	2.039	**0.044**
	C	E	-0.3669	0.1103	-3.327	**0.001**
adhesiveness	S	C	2.7483	1.3913	1.975	**0.050**
	C	E	-5.0066	2.0952	-2.39	**0.019**

Remarkably, at population scale, I found that both the locomotor performance traits differ significantly between north Sardinia (NS) and south Corsica (SC). The south Corsica population showed a better performance than that from north Sardinia by having a greater jumping force (SC=2.38 ± 0.062; NS=2.21 ± 0.059, p-value=0.045) as well as a higher adhesiveness force (SC=33.6 ± 1.3; NS=29.4 ± 1.22, p-value=0.019). Moreover, jumping force was significant different between north Sardinia and north Corsica (NS=2.21 ± 0.059; NC=2.5 ± 0.067, p-value=0.001) and, within Sardinia, between its south population (SS= 2.45 ± 0.077; NS= 2.21 ± 0.059, p-value=0.014) and north population. On the other hand, I did not find

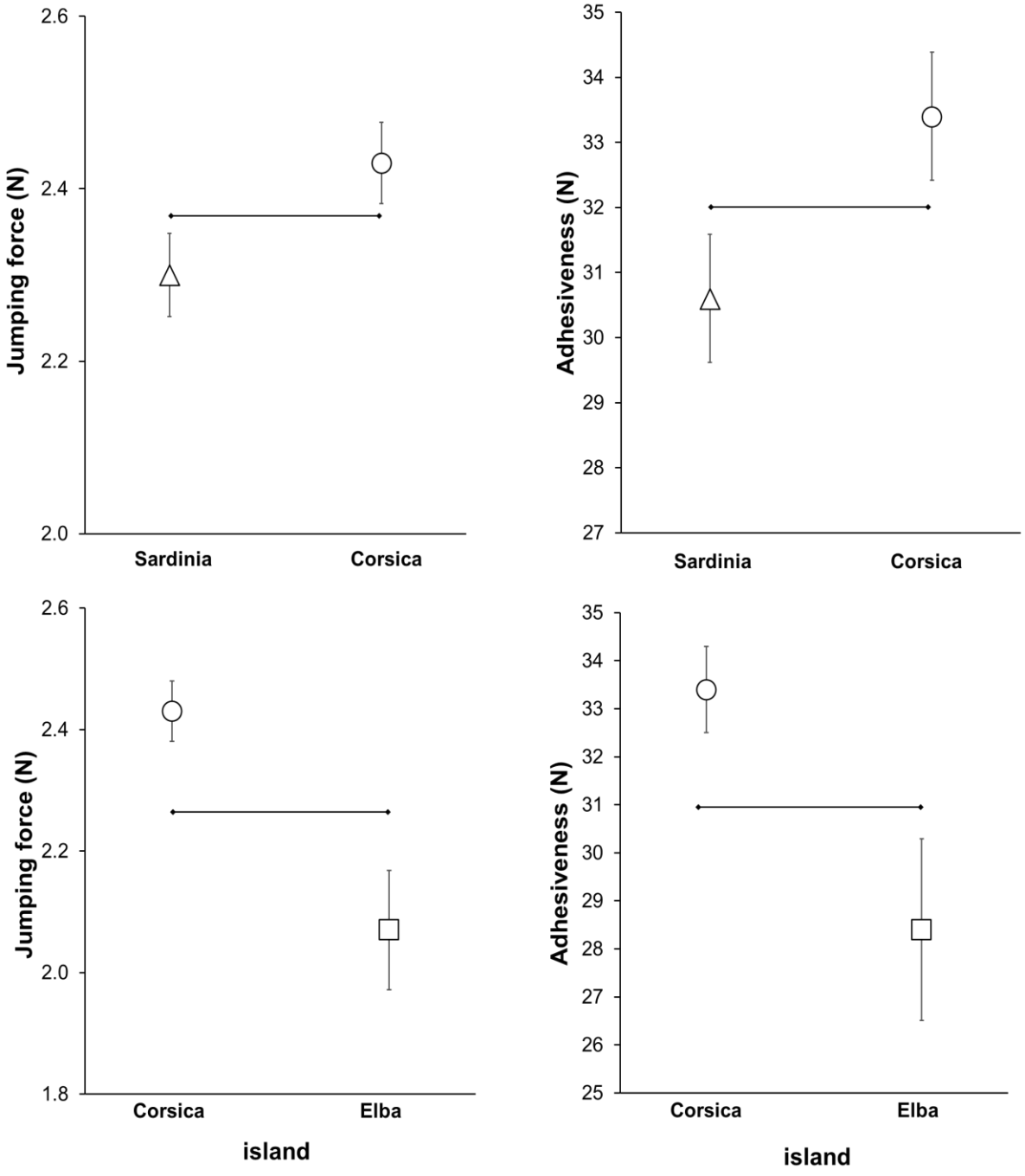
performance differences within Corsica. A summary of the coefficient estimates used to investigate population differences in performance traits is reported in Table 2.

Table 2 Coefficient estimates (± SE) of LMs with locomotory performance traits as dependent variable and Sardinia-Corsica populations as fixed factors. SS=South Sardinia; NS=North Sardinia; SC=South Corsica; NC=North Corsica. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	Ps(>|t|)
jumping force	SS	NS	-0.2411	0.0971	-2.484	**0.0145**
	SS	SC	-0.0672	0.0988	-0.681	0.4974
	SS	NC	0.0500	0.1023	0.489	0.6260
	SC	NC	0.1173	0.0919	1.276	0.2047
	SC	NS	-0.1739	0.0860	-2.022	**0.0456**
	NC	NS	-0.2912	0.0901	-3.232	**0.0016**
adhesiveness	SS	NS	-3.4755	2.0419	-1.702	0.0918
	SS	SC	0.7618	2.0901	0.364	0.7163
	SS	NC	0.2016	2.2100	0.091	0.9275
	SC	NC	-0.5601	1.9757	-0.284	0.7774
	SC	NS	-4.2373	1.7856	-2.373	**0.0195**
	NC	NS	-3.6772	1.9246	-1.911	0.0589

I found marked differences in both the locomotory performance traits between Corsica and Elba island (Table 1). Specifically, both the measured performance traits were higher in Corsica than in the Elba (jumping force: C=2.43 ± 0.049; E=2.07 ± 0.098, p-value=0.001, adhesiveness: C=33.4 ± 0.984; E=28.4 ± 1.89, p-value=0.019). The EMMs values for locomotory performance differences between Sardinia and Corsica and between Corsica and Elba reported in Figure 1.

Figure 1 EMMs and standard errors from LMs for performance traits of Sardinia-Corsica (on top) and Corsica-Elba (on bottom). S=Sardinia (triangle), C=Corsica (circle) and E=Elba island (square). Jumping force= maximum jumping force at take-off (N); Adhesiveness= maximum adhesion force (N). Significant post-hoc (Tukey) contrasts are indicated with a bar.



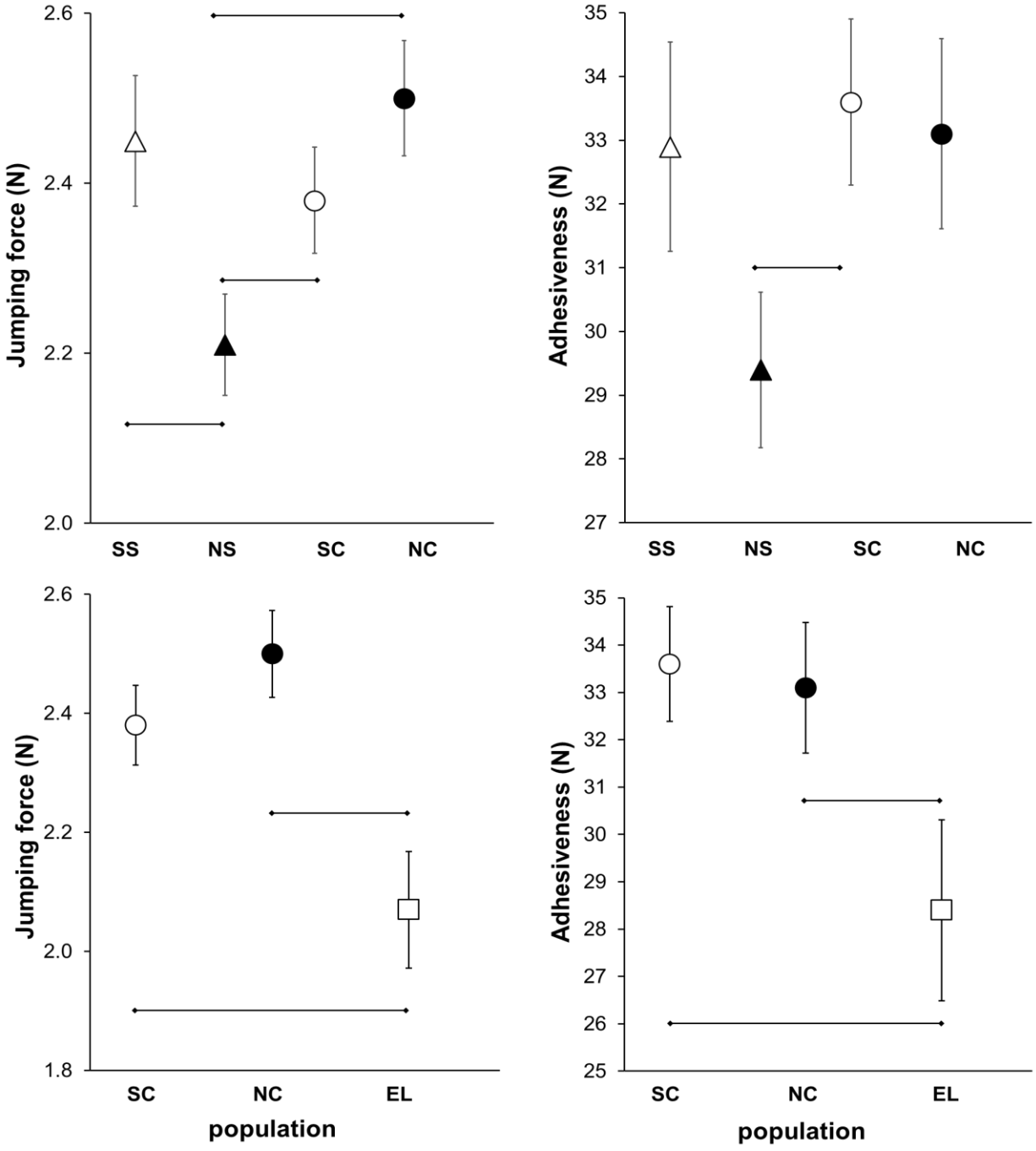
Both Corsica populations showed significant better performance as compared to population from Elba. The EMMs values of jumping force were higher in the north Corsica compared to those of Elba (NC=2.5 ± 0.073; EL=2.07 ± 0.098, p-value=0.0007), while those of the south Corsica lied in the middle (CS=2.38 ± 0.067, p-value=0.014). Similarly, the adhesiveness, was

significantly higher in Corsica (CS=33.6 ± 1.21; CN=31.1 ± 1.38) than in Elba (EL=28.4 ± 1.91). A summary of the coefficient estimates used to investigate population differences in performance traits is reported in Table 3. The EMMs values for locomotory performance differences among populations of Sardinia-Corsica and Corsica-Elba reported in Figure 2.

Table 3 Coefficient estimates (± SE) of LMs with locomotory performance traits as dependent variable and Corsica-Elba populations as fixed factors. SC=South Corsica; NC=North Corsica; EL=Elba. Significant contrasts are shown in bold.

Dependent variable	Reference Level	Level	Coefficient	SE	t value	Ps(>|t|)
jumping force	NC	SC	-0.1173	0.0994	-1.180	0.2421
	NC	EL	-0.4305	0.1225	-3.515	**0.0007**
	SC	EL	-0.3133	0.1190	-2.632	**0.0104**
adhesiveness	NC	SC	0.5601	1.8296	0.306	0.7605
	NC	EL	-4.6895	2.3509	-1.995	**0.0505**
	SC	EL	-5.2497	2.2549	-2.328	**0.0232**

Figure 2 EMMs and standard errors from LMs for performance traits among Sardinia-Corsica (on top) and Corsica-Elba (on bottom) populations. SS=South Sardinia (white triangle); NS=North Sardinia (black triangle); SC=South Corsica (white circle); NC= North Corsica (black circle); EL=Elba (white square). Significant post-hoc (Tukey) contrasts are indicated with a bar.



3.4 Discussion

In this study, I investigated if historical biogeographic processes might have produced a differentiation in locomotory performance traits among three islands (Sardinia, Corsica and Elba island) distributed along the inferred route of past *H. sarda* range expansion. To test a differentiation in locomotory performance traits as a consequence of a Pleistocene range expansion I compared Sardinia with Corsica, while the outcomes of a past island colonization were investigated by comparing the same traits between Corsica and Elba island. Below, I discussed the results from these two comparisons (Sardinia *vs.* Corsica and Corsica *vs*. Elba island) separately.

3.4.1 Historical biogeography of locomotion: range expansion through spatial diffusion

Here, I provide evidence of a significant differentiation in both the locomotory performance traits along the Sardinia-Corsica route of the *H. sarda* past expansion. I found that tree frogs from Corsica showed a better locomotory performance than their ancestors from Sardinia by having greater jumping force at take-off, as well as higher adhesion force. Notably, the geographic pattern of the jumping force seems to retrace the route of the past expansion with growing values starting from the north Sardinia up to the north of Corsica. Moreover, both locomotory performance traits showed substantial differences between northern Sardinia and southern Corsica, in coincidence with the wide and suitable land-bridge area that connected the two islands at time of past expansion (Thiede, 1978).

These patterns suggest that those individuals that arrived in Corsica, crossing the land bridge, may have been a non-random sample of "good performer" phenotypes of the ancestral population (Sardinia). A possible scenario is that only those individuals carrying "good" locomotory traits might have been able to successfully arrive in Corsica. Locomotory performance traits are indeed directly implicated in dispersal processes and, therefore, most

likely influenced by an expansion process (Canestrelli et al., 2016a, b). In an arboreal species such as the tree frog, both the jumping force at take-off as well as the adhesiveness are key traits in determining its dispersal ability (Duellman and Trueb, 1994) and, therefore, expected to be under strong selection during an expansion process.

Alternatively, it could be that tree frogs were spatially sorted according to their dispersal ability by the past expansion process (Phillips et al., 2010; Shine et al., 2011). This could have led to a rapid and directional evolution in locomotory performance traits along the south-north expansion axis, possibly favouring the formation of the current geographical patterns. Recent evidence showed the evolution of locomotory performance traits at the range-edge of expanding population (Phillips et al., 2008, Alford et al., 2009; Louppe et al., 2017). For example, in the cane toad (*Rhinella marina*), a fast dispersal rate with straighter paths and even higher endurance has been documented at the edge of invaded Australian range (Phillips et al., 2008; Alford et al., 2009; Llewellyn et al., 2010). Similarly, we found that dispersers at the range-edge (Corsica) have a higher jumping force at take-off, as well as a higher adhesion force than those living in the ancestral areas (Sardinia). Hence, our results are in line with previous study on the locomotory performance traits in expanding populations (Phillips et al., 2008; Alford et al., 2009; Llewellyn et al., 2010).

However, to the best of my knowledge, this is the first study to indicate a directional evolution in locomotory performance traits from an historical range expansion. Thus, my results suggest that a historical expansion process may have contributed to shape the species geographic variation in phenotypic traits that are crucial for species dispersal ability.

3.4.2 Historical biogeography of locomotion: island colonization through jump dispersal

Here, I provide evidence of a significant differentiation in both the locomotory performance traits across Corsica and Elba populations. Tree frogs from Corsica showed a better locomotory performance than those living on Elba, by having greater jumping force at take-off as well as

higher adhesion force. The origin of such differentiation between the two islands could be the result of a past founder event, as supported by the low genetic diversity characterizing Elba if compared with the rest of species' range (Bisconti et al., 2011b). The founder event could have contributed to fix such locomotory traits which would have become highly heritable. Moreover, the isolation may have precluded the introduction of 'fresh' alleles thus preventing the evolution of such traits over time.

Alternatively, but this explanation is not mutually exclusive with the previous one, the persistence of such differences in locomotory traits would be explained by the adaptation to the relaxed ecological conditions generally occurring on islands (e.g. rodents: Adler and Levins, 1994; birds: Blondel, 2000; reptiles: Novosolov et al., 2013). The ecological relaxation has indeed been associated with the loss of costly antipredator "machinery", including performance traits (MacArthur and Wilson, 1967; Blumstein et al., 2002; Vervust et al., 2007). Vervust et al. (2007) found that lizards inhabiting a more "dangerous" island, in terms of predation pressure, showed a higher sprint speed capacity and flee faster and farther than lizards living in the safer island. Although we have no information about predation pressure in Elba island, assuming a trend similar to most islands it is possible that costly locomotory performance may have been counter-selected by the ecological relaxation, by determining the lower locomotory performance (Blumstein et al., 2002; Blumstein et al., 2005).

CHAPTER 4 PHYSIOLOGICAL TRAITS

4.1 Introduction

Aging, the physiological decline associated with advancing age, is a ubiquitous process in nature (Froy and Gamelon, 2020). Research on laboratory animal models showed that the regulation of oxidative status is one main physiological mechanism of the rate of ageing (Partridge and Gems, 2002). On the other hand, we still know relatively little about age-related changes in oxidative status markers in in free-living animals or non-model laboratory organisms (Gaillard and Lemaître, 2020).

This research gap is surprising if we consider that the expression of oxidative status traits might integrate the individual history or genetic background and underlie life-history trade-offs (Costantini, 2014). For example, variation of oxidative status markers may be linked to immune function (Sorci and Faivre, 2009), personality traits (Herborn et al., 2011), chronic stress exposure (Hau et al., 2015) or demographic traits (Costantini et al., 2015). This emerging view of oxidative status biology as an integrative research ground, suggests potential implications of physiological ageing in eco-evolutionary processes, such as dispersal, that mould the integrative phenotypes.

Dispersal is a key biogeographic context-dependent process that contributes to shape spatial dimension of biodiversity (Hanski and Gilpin, 1991; Hansson, 1991; Bohonak, 1999; Clobert, 2001; Ronce, 2007; Saastamoinen et al., 2018). Recent theoretical and empirical evidences suggest that dispersal could also depend on individual state or phenotypic quality, which can either influence or be influenced by the evolution of virtually all the features of an organism, including physiology (Tracy et al., 2012; Rollins et al., 2015; Canestrelli et al., 2016b). Dispersal is a highly energetically and resource demanding process (Bonte et al., 2012). It imposes a number of physiological challenges, which are expected to vary greatly between

individuals living at the edge and those living at the core of an expanding populations. Individuals at the edge of an expanding population are likely to experience novel ecological pressures resulting from new predators, pathogens, or prey (Sakai et al., 2001; Lee and Klasing, 2004; Brown et al., 2013). Additional challenges derive from the new abiotic conditions to cope with, such as temperature and humidity. Temperature can constrain or enhance dispersal activity by its effect on physiological functions (Wilson et al., 2000; Johnston and Temple, 2002; Steinhausen et al., 2008), especially in ectotherms. Thus, dispersers are required to acclimatise physiologically to the changing environmental conditions. One main consequence of this physiological plasticity might lie with a differential allocation of resources between dispersal and life-history traits (Hughes et al., 2003; Burton et al., 2010; Bonte et al., 2012; Brown et al., 2015; Hudson et al., 2015; Chuang and Peterson, 2016). For example, individuals at range edges tend to grow faster (invertebrates: Therry et al., 2014; fish: Bøhn et al., 2004; Myles-Gonzalez et al., 2015; amphibians: Phillips, 2009; Lindström et al., 2013) and to invest less in reproduction (invertebrates: Hughes et al., 2003; amphibians: Hudson et al., 2015; Courant et al., 2017, but see Hanski et al., 2006) than those from the origin areas.

Recently, some appealing eco-immunological theories have explored the idea that dispersal may also result in a reduced investment in immune function (Lee and Klasing, 2004; White and Perkins, 2012; Cornet et al., 2016), at least in invasive species. Empirical evidence of changes in immune investment has been observed at the edge of invasive populations (Lee, Martin and Wikelski, 2005; Brown and Shine, 2014; Cornet et al., 2016; Goetz et al., 2017). There are three reasons to expect benefits from a reduced immune investment during invasion. First, many co-evolved native pathogens may be lost (‘enemy release hypothesis’ Colautti et al., 2004) during dispersal as proved in many animal species (Philips et al., 2010; Gendron et al., 2012). Second, the ‘new’ pathogens should be less virulent because they lack of a co-evolutionary history with the ‘new’ host (Lee, Martin and Wikelski, 2005). Third, the energy trade-off between immunity and strenuous muscular activity is expect to be directed towards the last, resulting in down-

regulation of the immune system in order to counteract the metabolic consequences of muscular activity, such as generation of free radicals (Martin et al., 2012; White and Perkins, 2012; Brown and Shine, 2014).

The outcome of an invasion/expansion also depends to a large extent on aerobic (oxidative) metabolism (Seebacher and Franklin, 2011) required to energetically support dispersal activity itself. Thus, metabolic constrains could play a crucial role in shaping trade-offs between dispersal and life-history strategies (Tracy et al., 2012; Myles-Gonzalez et al., 2015; Louppe et al., 2018). In this context, studies led to controversial results possibly due to the differences in the used methods or in the considered species. In fact, no differences in standard metabolic rate were found between edge and core populations of *Rhinella marina* (Tracy et al., 2012), while significant differences were found in the *Neogobius melanostomus* showing higher standard metabolic rate at the edge while (Myles-Gonzalez et al., 2015), in the *Xenopus laevis* the opposite pattern was found (Louppe et al., 2018). To date, the metabolic constraints that underlie life-history variation in relation to dispersal remain unclear. Importantly, both metabolic rate and immune function may affect certain traits of oxidative status (Costantini, 2014), strengthening the idea that oxidative status and its role in physiological ageing might vary among populations across an expansion gradient.

Metabolic activity is indeed a continuous source of highly unstable oxidant chemicals, such as the reactive oxygen species (ROS). Organisms have evolved a complex network of molecular mechanisms to regulate the oxidant-antioxidant homeostasis (Costantini, 2008, 2019). For example, animals rely on a number of antioxidant molecules to prevent damage or remove accumulation of pro-oxidant compounds. This antioxidant system includes enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) or glutathione reductase (GR), and low molecular non-enzymatic molecules, such as glutathione (GSH) or vitamins. Investment in antioxidant expression and detoxification from ROS to control generation of molecular oxidative damages is costly for the organism, thus we might expect

that, during dispersal, individuals at edge are differently exposed to oxidative stress compared to those at the core as a response to a differential metabolic requirements and allocation trade-offs (Myles-Gonzalez et al., 2015; Louppe et al., 2018).

The regulation of oxidative status is also expected to have an important role during dispersal because of its intimate links with some components of the immune function (Costantini and Møller, 2009; Sorci and Faivre, 2009). The change in oxidative status following an immune response is highly conserved across animals, from invertebrates (Philipp et al., 2011; Genard et al., 2013) to mammals (Schneeberger et al., 2013; Finotello et al., 2014). This is because immune cells (phagocytes and lymphocytes) kill pathogens by releasing lethal pro-oxidant compounds (Babior, 2004; Segal, 2005; Halliwell, 2006) causing oxidative stress (Costantini and Møller, 2009). Moreover, immune responses are characterised by increased metabolic activity (e.g., energy expenditure), which can further contribute to ROS production. Overall, these evidences suggest that regulation of oxidative status might play a significant role in the dispersal process, but so far none studies have tested this hypothesis.

In this study, I have tested for the first time whether dispersal-driven processes of historical biogeographic relevance, such as range expansions, have moulded physiological ageing rates by leaving detectable imprints of this influence on age-related changes in oxidative status markers. My main hypothesis is that, if individual variation in physiological ageing reflects the variation in the capacity to cope with the new demographic and/or environmental conditions encountered during a range expansion, a spatial structure in oxidative status might emerge along the range of a recently expanded population.

I expect that individuals at the edge (Corsica) may show a higher pace of physiological ageing then their ancestors from Sardinia. A common garden longitudinal experiment (i.e., with a repeated measurement design) was used to test the hypothesis that the expansion process induced a differentiation in oxidative status between tree frogs (*H. sarda*) from Sardinia (core) and Corsica (edge) islands. I investigated differences in four blood-based markers of oxidative

status: primary oxidative damage, measured as reactive oxygen metabolites (ROMs) in serum; serum non-enzymatic antioxidant capacity, estimated through the OXY test; the enzymatic antioxidant activity in erythrocytes of glutathione peroxidase (GPx) and superoxide dismutase (SOD). I also calculated the within-individual repeatability over the experiment of the four markers in order to assess the extent to which the oxidative status is constrained by endogenous individual features.

4.2 Materials and Methods

I collected and housed individuals following the same procedure reported above (see chapter 2.2.1). I used a total of 55 individuals, from Sardinia and Corsica, for the measurement of the oxidative biomarkers. I excluded individuals from Elba island from blood sampling because several of them were too small and emaciated and their removal would lower sample size.

4.2.1 Blood sampling

Blood samples were collected from 55 individuals (32 from Sardinia, 23 from Corsica) and, one year later, another sample of blood was collected from 46 individuals still alive (31 from Sardinia, 15 from Corsica) at CISMAR laboratories. To collect blood, I gently took individuals from their home cage and I anesthetized by immersing them for two minutes in a solution of MS-222 (Tricaine methanesulfonate, 0.5 % m/v). After that, they underwent an intracardiac injection with a 0.3 ml syringe. I collected about 50-100 µL of blood from each individual, I placed it in rack kept at 4°C, and within few minutes I centrifuged it (Sigma centrifuge 1-14) at 10000 rpm for 3 minutes. Subsequently, I processed each sample in order to separate the serum from the erythrocytes in two different tubes that I immediately stored at -80 C° until laboratory analyses. After blood collection, I placed each individual in a single-use plastic box containing dechlorinated water and I monitored until it was fully awakened. After that, I gently returned back each individual to its home cage. I carried out laboratory analyses within five months from blood collection at the MNHN (UMR7221) of Paris.

4.2.2 Laboratory analyses

I measured four markers of oxidative status according to established protocols for vertebrates (Costantini et al., 2011). Specifically, as marker of oxidative damage I measured using the Reactive Oxygen Metabolites assay (d-ROMs test, Diacron International, Grosseto, Italy). The

d-ROMs assay measures the serum metabolites of oxidative damage, mainly hydroperoxides. Hydroperoxides are compounds generated early in the oxidative cascade from the oxidation of several molecular substrates, such as fatty acids, proteins and nucleic acids. Under the d-ROMs test conditions, the hydroperoxides contained in the sample undergo a Fenton reaction generating in vitro through their breakage the peroxyl (R-OO·) and alkoxy (R-O·) radicals that react with a chromogen solution (diethyl p-phenylene diamine hydrochloride). The ROMs concentration correlates directly with the colour intensity detected by photometric reading. The ROMs concentration is expressed as mM of H_2O_2 equivalents. I carried out the reactive oxygen metabolites (d-ROMs) assay (Diacron International, Grosseto, Italy) with some modifications respect to the manufacturer's instruction. Reactions were run in 96-well plates; in each plate, I tested a calibration curve (including blank) using the reference standard supplied with the assay, a quality control (i.e., a serum sample with a known concentration supplied with the assay) and serum samples in duplicate. I used a volume of 4 µl for all samples, standards and quality controls. Absorbance was read at a wavelength of 492 nm ± 10 nm and at the reference wavelength of 612 nm using ULTRA evolution plate reader (Tecan MDCL, Hamilton, Canada). Analyses were run in duplicate; the mean coefficient of variation of serum samples was 8.82 % and of quality controls was 8.01 %.

I used the Oxy Adsorbent test (Diacron International, Grosseto, Italy) to evaluate the ability of serum non-enzymatic antioxidant compounds (vitamins, carotenoids, uric acid, thiol proteins etc.) to react in vitro with a known concentration of hypochlorous acid (HOCl). Unreacted HOCl react with the chromogen solution (*N*, *N*-diethyl-p-phenylendiamine) to form a coloured complex. The OXY Adsorbent test was performed following the manufacturer's instructions with some minor adjustments. An aliquot of 2 µl of the diluted serum (1:100 with distilled water), of reference standard, of blank (distilled water) and of quality control were incubated with 200 µl of HOCl solution for 10 minutes at 37°C. At the end of the incubation, 2µl of the chromogen was added to each well. The absorbance was read at a wavelength of 492 nm ± 10

nm and at the reference wavelength of 612 nm. Analyses were run in duplicate; the mean coefficient of variation of serum samples was 8.41 % and of quality controls was 4.64 %. OXY values were expressed as mM HOCl neutralised/mg proteins measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

I measured the activity of the antioxidant enzyme glutathione peroxidase (GPx) with the Ransel assay (Randox Laboratories, Crumlin, UK), in erythrocytes. The GPx is an enzyme that detoxifies cells from the accumulation of peroxides and organic hydroperoxides. In the Ransel assay, GPx catalyses the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. Under the assay conditions, the oxidised Glutathione (GSSG) is immediately converted to its reduced state. This last reaction is accompanied by a decrease in absorbance at 340 nm providing a spectrophotometric means for monitoring GPx activity. The Ransel assay was performed following the manufacturer's instructions with some modifications. An aliquot of 2µl of each haemolysate sample (obtained by diluting erythrocytes into distilled water) was used for the analyses. The absorbance of the reaction was red three times (after 1, 2 and 3 minutes) at a wavelength of 340 nm. The GPx activity was calculated following this formula [(Abs at 1 minutes – Abs at 3 minutes) / 2] x 15.873. Analyses were run in duplicate and the mean coefficient of variation was 6.88 %. The activity of GPx was expressed as Units/mg of proteins. measured using the Bradford protein assay.

I carried out the Ransod assay (Randox Laboratories, Crumlin, UK) to quantify the activity of the antioxidant enzyme superoxide dismutase (SOD) in erythrocytes. The SOD catalyses the dismutation of the superoxide radical (O2·) into molecular oxygen or hydrogen peroxide. The assay employs xanthine and xanthine oxidase (XOD) to generate the superoxide radical (O2·) which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The SOD activity is measured by the degree of inhibition of this reaction. One unit of SOD causes a 50% of reaction inhibition under the assay conditions. Some modifications to the manufacturer's instructions have been applied to the Ransod assay. An

aliquot of 6 µl of each haemolysate, 200 µl of Reagent 1 (Xanthine and I.N.T) and 30 µl of Reagent 2 (XOD) were used. The kinetic reaction was followed for 3 minutes and 30'' and the absorbance was red at a wavelength of 505 nm. The activity of SOD was expressed as Units/mg of proteins measured using the Bradford protein assay.

4.2.3 Data analyses

All statistical analyses were run using R (version 3.5.3; R Core Team 2019).

First, I performed Generalized Linear Mixed Models (lme4 package) with a repeated measures design to test whether the change of each marker over time differed between islands. In each model, I entered island (Sardinia *vs.* Corsica), sampling period (beginning and end of the experiment) and their interaction as fixed factors; individual was included as random factor to control for non-independence of repeated measurements. As dependent variables, I entered each marker singly; I also run a model using the SOD/GPx ratio as dependent variable because prior work suggested that an unbalanced ratio of the two enzymes might be associated with cellular ageing (De Haan et al., 1995). A Gamma distribution and a log link function was used for ROMs, OXY and SOD/ GPx, while a Gamma distribution and an identity link function was used for GPx and SOD.

Preliminary models showed that entering sampling location within each island as a random factor did not improve the fitting of the model (i.e., the Akaike Information Criterion value was not reduced beyond 2), thus it was no longer considered. Preliminary models also showed that the fitting of models based on the whole dataset was better than those of models based on a subset of individuals of which we had data for both the pre- and post-experimental period (data not shown).

I ran Generalized linear mixed effect (GLMM)-based repeatability models (glmer in package 'lme4') to estimate the within-individual repeatability of all oxidative status markers. Gamma

distribution with a log link function was used. The observation-level variance was obtained by using the trigamma function (Nakagawa et al., 2017). I entered each marker singly as a dependent variable and individual as random factor. Preliminary models showed that the adjusted repeatability calculated by entering location as fixed factor did not improve the fitting of the models.

Finally, I compared the mortality percentage observed from the first to the second sampling of blood between islands using p-value = $(p_s \times n_s + p_c \times n_c)/(n_s + n_c)$ in STATISTICA (Version 10 StatSoft, Tulsa, OK, USA), where p is the proportion of dead frogs, n is the sample size, s is Sardinia and c is Corsica.

I also tested whether there was any selective disappearance between the first and second sampling period of individuals from a given island having different values of each markers at the beginning of the experiment as compared to the overall distribution. I ran a Generalized Linear Models (glm in package 'lme4') with a binomial error distribution and a logit link function that included a given marker as main factor and survival probability (0 = dead, 1 = alive) until the end of the experiment as response variable. These analyses were restricted to Corsica because only one frog from Sardinia died before the second sampling of blood.

4.3 Results

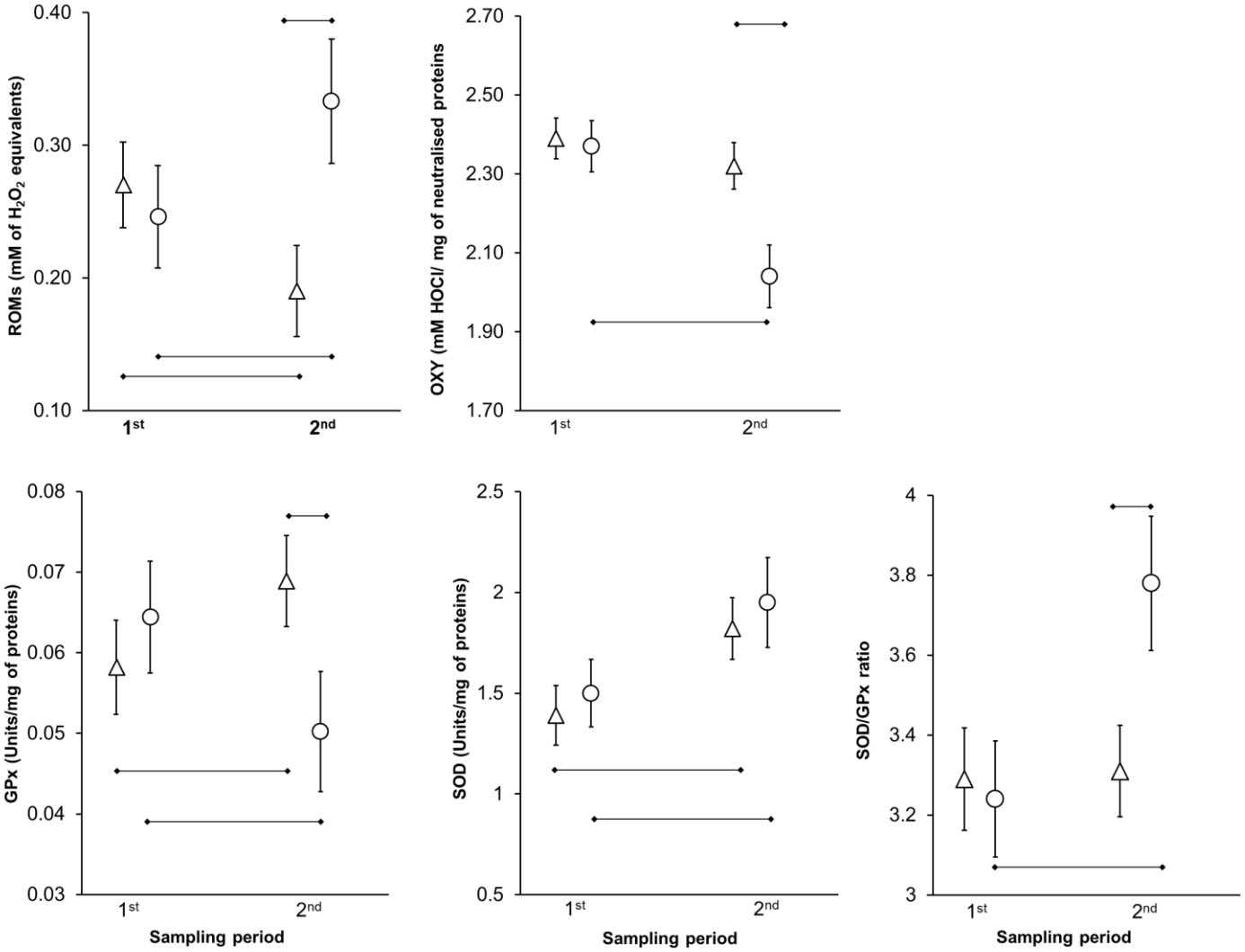
I found a significant interaction between island and sampling period for ROMs, OXY, GPx and SOD/GPx ratio (Table 1).

Table 1 Summary of GLMMs. Markers were entered as dependent variables; island, sampling period and their interaction were entered as fixed factors; individual identity was included as a random factor. Significant factors are shown in bold.

Variable	Effect	F-value	p-value
ROMs	island	1.05	0.630
	sampling	0.86	**0.028**
	island x sampling	9.09	**0.001**
OXY	island	2.99	0.745
	sampling	11.20	**<0.0001**
	island x sampling	6.11	**0.010**
GPx	island	0.72	0.484
	sampling	0.28	**0.015**
	island x sampling	8.35	**0.001**
SOD	island	0.04	0.606
	sampling	11.32	**0.038**
	island x sampling	0.002	0.954
SOD/GPx	island	1.11	0.824
	sampling	4.46	**0.002**
	island x sampling	6.12	**0.013**

Post-hoc analyses by Tukey test showed that islands had similar values of all tested markers at the beginning of the experiment. Conversely, at the end of the experiment, Corsica tree frogs had significantly higher ROMs ($p=0.013$) and lower OXY ($p=0.005$) and GPx ($p=0.038$) than those from Sardinia. The changing in the islands' oxidative status for both sampling period are reported as EMMs ± SE from GLMMs in Figure 1.

Figure 1 EMMs and standard errors from GLMMs for oxidative markers between Sardinia (triangle) and Corsica (circle). ROMs =reactive oxygen metabolites in serum; OXY=serum non-enzymatic antioxidant capacity; GPx=glutathione peroxidase; SOD=superoxide dismutase; GPx/SOD=ratio between GPx and SOD. Significant post-hoc (Tukey) contrasts are indicated with a bar.



From the beginning to the end of the experiment, ROMs decreased in Sardinia (p=0.003) and increased in Corsica (p=0.028). On the other hand, GPx, significantly increased in Sardinia (p=0.007) and decreased in Corsica (p=0.015). OXY did not change in Sardinia over the experiment, while it decreased significantly in Corsica (p<0.0001). SOD increased significantly in both Sardinia (p=0.006) and Corsica (p=0.038) over the course of the experiment; the SOD/GPx increased significantly in Corsica frogs (p=0.007). Overall, at the end of the

experiment, tree frogs from Corsica showed higher ROMs and lower values of OXY, GPx and SOD/GPx than those from Sardinia.

All markers of oxidative status were significantly repeatable over the course of the experiment; estimates of repeatability coefficients varied from 0.25 for OXY to 0.48 for GPx (Table 2).

Table 2 Summary of GLMM-based repeatability estimates for Sardinia *vs*. Corsica. Markers were included in the models as dependent variables and the individual was entered as a random factor. Gamma distribution with log link function was used. The observation-level variance was obtained by using the trigamma function in R.

Variable	Estimate	Observation-leval variance
ROMs	0.40	0.0168
OXY	0.25	0.0607
GPx	0.48	0.1392
SOD	0.26	0.1598
SOD/GPx	0.35	0.2163

I recorded a higher mortality of frogs from Corsica (8 out of 23, 34.8%) than those from Sardinia (1 out of 32, 3.1%; p=0.002). Values of each marker at the first sampling of blood did not predict the mortality probability until the second sampling of blood in frogs from Corsica (p-values≥0.41), indicating that results were robust for any potential bias introduced by selective mortality.

4.4 Discussion

In this study, I investigated whether core (hereafter Sardinia) and edge (hereafter Corsica) populations of *Hyla sarda* differed in the age-related oxidative status markers as a consequence of a past range expansion. Using a longitudinal common garden experiment, I provide compelling evidence of a significant differentiation in the oxidative status among populations of tree frogs distributed along an historical range expansion route from Sardinia to Corsica. At the beginning of the experiment, Sardinia and Corsica frogs showed similar concentrations of all oxidative status markers, which means that the oxidative status was not affected by the different ecological conditions occurred in the sampling areas. In contrast, one year later (i.e., at the end of the experiment), Sardinia and Corsica frogs had sharp differences in most markers analysed. Specifically, frogs from Corsica had significantly higher ROMs (marker of oxidative damage) and lower antioxidant molecules (OXY and GPx) than those from Sardinia. The SOD/GPx ratio was also significantly higher in Corsica than in Sardinia frogs. In contrast, I found that expression of the antioxidant enzyme SOD was increased at the end of the experiment in both islands. Notably, I also found that frogs from Corsica suffered higher mortality than frogs from Sardinia over the course of the experiment. Overall, these results point to a differentiation in the physiological ageing between conspecific populations that have been geographically and genetically isolated since the end of the Pleistocene epoch (Bisconti et al., 2011a). These results were robust for any selective mortality that occurred during the experiment. Moreover, as frogs were maintained under the same common garden conditions, I can discard any bias owing to different maintenance conditions.

An explanation for these results might lie with variation between islands in the endogenous production of reactive oxygen species Mitochondrial activity, is one important source of the free radical superoxide anion that, in turn, is precursor of other pro-oxidants, such as hydrogen peroxide (Halliwell and Gutteridge, 2015). SOD, an enzyme that protects against the superoxide

anion, was upregulated over the course of the experiment in both islands. Thus, we might exclude a strong role of mitochondrial superoxide anion in explaining our results if the level of SOD expressed in the two islands was similarly effective in controlling pro-oxidant activity of superoxide anion.

Alternatively, Sardinia and Corsica frogs might have differed in the activity of the hypothalamic-pituitary-interrenal axis that releases corticosterone (Sapolsky et al., 2000; Romero, 2004). Corticosterone is an important regulators of energy balance involved in the mobilization of stored energy essential for self-maintenance and survival (McEwen and Stellar 1993; Wingfield et al., 1998). In fact, corticosterone promotes a suite of metabolic processes such as the increase gluconeogenesis, catabolism of energy reserves, inhibition of energy storage, and increase in locomotor and foraging activity (Sapolsky et al., 2000). However, corticosterone can also induce changes in the oxidative status, especially when the animals are chronically exposed to increased levels of corticosterone for long period of times (Costantini et al., 2011). In the house sparrow (*Passer domesticus*) a differential regulation of corticosterone was observed across its invaded Kenyan range (Liebl and Martin, 2012, 2013). Individuals from range-edge release more corticosterone possibly in response to their exposure to novel stressful environmental stimuli. Corticosterone has long also been considered a promotor of dispersal by stimulating activity in birds and, recently, in movement-related survival in cane toads (Belthoff and Dufty, 1998; Jessop et al., 2018). Moreover, a recent experiment of substrate choice suggests that corticosterone levels may indicate matrix resistance in common toad (*Bufo bufo*), thus providing an estimate of physiological cost to animals migrating through different matrixes (Janin et al., 2012) such as those encountered during an expansion. Further work will be needed to elucidate the role of corticosterone as a pacemaker of age-related changes of oxidative status in tree frogs.

The results of my work raise the important question of why these population differences in oxidative status have evolved. An explanation might lie with the legacy of a past positive

selection on those life-history strategies that were more successful during the range expansion. Notably, a substantial upregulation of genes involved in metabolism and cellular repair, including mechanism of response to oxidative stress, was found at the edge of the invasion front of cane toad (Rollins et al., 2015). These results suggest that dispersing cane toads might have prioritized dispersal activity rather than self-maintenance mechanisms. This strategy would be successful whether the costs of reducing protection against oxidative damages are low as compared to those incurred to sustain active defence mechanisms. This explanation is congruent with the disposable soma hypothesis of ageing, which states that optimisation of trade-offs in the allocation of limited resources among self-maintenance and other activities inevitably comes at a cost for the soma in the long-term (Kirkwood, 1977; Kirkwood and Rose, 1991). Similarly, the antagonistic pleiotropy hypothesis of ageing posits that natural selection would favour the evolution of life histories in which alleles have beneficial effects on fitness early in life even when they are detrimental in old individuals (Williams, 1957; Nussey et al., 2013). The cost of ageing would be low because it would emerge at a phase of lifetime (old age) when the force of natural selection is expected to be low as in the case of our frogs. Our longitudinal data actually covered a non-negligible portion of the expected lifespan of a treefrog (one out of approximately three years; Cadeddu et al., 2012). The selection pressures produced by extrinsic mortality factors are thought to play a major role in the evolution of ageing rates (Monaghan et al., 2008). The risk of death from extrinsic causes, such as disease or predation, during dispersal is particularly important (Clobert et al., 2012). Investing resources in a robust and long-lasting body might be maladaptive if extrinsic causes of mortality are high. In these circumstances a much better strategy, in evolutionary terms, would be to invest more resources into traits that favour dispersal. Thus, the high oxidative stress and mortality rate found in Corsica tree frogs may represent an imprint of physiological cost to maintain dispersal traits useful to successfully colonize the northern portion of their range.

Finally, I found a moderate individual repeatability of all measured markers (0.25 to 0.48). Estimates of within-individual repeatability of oxidative status markers are rarely reported in the literature and the few available are in line with our estimates (e.g., Saino et al., 2011; Récapet et al., 2019). The mechanistic reasons for and biological meaning of these intrinsic individual differences in oxidative status markers have not been elucidated yet.

In conclusion, my study provides strong evidence for a role of adaptive processes that occurred during an historical range expansion in explaining the differential physiological ageing among populations of tree frogs. The results also indicate that, while age-related changes in SOD might be a shared (or conserved) mechanism (*sensu* Partridge and Gems, 2002) of ageing, those in other oxidative status markers (ROMs, serum non-enzymatic antioxidants and GPx) might be private (peculiar to particular evolutionary lineages) because they were expressed only in Corsica frogs. The possible conserved SOD expression across the two islands is also supported by the lack of genetic variation between Sardinia and Corsica frogs in the gene Sod-1 (Bisconti et al., 2011b). Further studies will be needed to elucidate the endogenous mechanisms responsible for the observed differences in oxidative status and the correlated functional and fitness consequences. It will also be important to determine whether such differences are owing to genetic variants or developmental plasticity.

CHAPTER 5 TELOMERE LENGTH

5.1 Introduction

Telomeres are ancient and highly conserved nucleoprotein structures located at the terminal ends of eukaryote chromosomes (Blackburn and Gall, 1978). These strings of a repeated, short, non-coding sequence of DNA (Gomes et al., 2010) play a major role in maintaining chromosome and genome stability and in avoiding loss of genetic material (Blackburn, 2005; O'Sullivan and Karlseder, 2010). Although can be restored by the enzyme telomerase at each replication cycle (Greider and Blackburn, 1989), telomeres shorten over time, and are therefore considered a molecular marker of cellular ageing (Harley, 1991; Counter 1996). Because of this potential link with longevity, recently there has been a growing interest in understanding the biological meaning of variation in telomere length and dynamics (Monaghan et al., 2018). Telomere length variation has indeed been implicated in several processes of ecological importance in a wide range of organisms. For example, telomere dynamics have been found affected by habitat quality in the American redstart (*Setophaga ruticilla*). Specifically, telomeres of males wintering in a nonbreeding low-quality habitat shorten faster than those of individuals wintering in a high-quality habitat (Angelier et al., 2013). Moreover, the early-life adversity, such as brood intraspecific competition, has been associated with relatively short telomeres, at least in several avian species (Angelier et al., 2018 and reference therein). The rate of telomere shortening may also be increased by chronic stress exposure (Hau et al., 2015). The effects of chronic stress on telomeres length was observed by Hau et al. (2015) in adult of Eurasian blackbirds (*Turdus merula*) that were exposed to a repeated disturbance and immune stressors over time. The immune function has also been associated with reduced telomere length in several species (Ilmonen et al., 2008; Asghar et al., 2015a; Georgin-Lavialle et al., 2010). In wild-derived mice (*Mus musculus*) a rapid telomere attrition was found in response to a repeated bacteria exposure (Ilmonen et al., 2008) while, in the great reed warbler (*Acrocephalus*

arundinaceus) chronic malaria infection was associated with significantly faster telomere attrition (Asghar et al., 2015a). Furthermore, telomere dynamics have been associated with personality traits in the brown trout (*Salmo trutta*), whose individuals with shorter telomeres behave consistently more boldly and aggressively, suggesting that telomere dynamics may be an important state variable in the regulation of personality (Adriaenssens et al., 2016). These findings highlight that telomere length might represent a valuable metric of individual phenotypic quality (Angelier et al., 2019), suggesting its potential implications in processes of eco-evolutionary importance, such as dispersal.

Dispersal is a key process in ecology and evolution because of its consequences for demography and gene flow (Hanski and Gilpin, 1991; Hansson, 1991; Bohonak, 1999; Clobert, 2001; Saastamoinen et al., 2018). Dispersal is as an essentially condition-dependent process (Bowler and Benton, 2005; Clobert et al., 2009; Lowe et al., 2014; Cote et al., 2017) that can influence - and be influenced by - the evolution of virtually all the features of an organism, including behaviour (Bowler and Benton, 2005; Clobert et al., 2012; Chaine and Clobert, 2015; Canestrelli et al., 2016a), locomotory performance (Phillips, 2008; Alford et al., 2009; Louppe et al., 2017) and physiology (Tracy et al., 2012; Rollins et al., 2015; Canestrelli et al., 2016b).

In this study, I have test for the first time whether dispersal-driven processes of historical biogeographic relevance, such as range expansions, can affect telomere length and telomere dynamics and leave detectable imprints of this influence. My main hypothesis is that, if individual variation in telomere dynamics translates into variation in the capacity to cope with the new demographic and/or environmental conditions encountered during a range expansion, a spatial structure in telomere dynamics might emerge along the range of a recently expanded population.

I used a long-term longitudinal common garden experiment (i.e., with a repeated measurement design) to test the hypothesis that the expansion process induced a differentiation in telomeres

length and its change over time between tree frogs (*H. sarda*) from Sardinia (core) and Corsica (edge) islands.

5.2 Materials and Methods

I collected and housed individuals following the same procedure reported above (see chapter 2.2.1). I used a total of 47 individuals, from Sardinia and Corsica, for the measurement of the telomere length and its change over time. I excluded individuals from Elba island from blood sampling because several of them were too small and emaciated and their removal would lower sample size.

5.2.1 Blood sampling

Blood was collected following the same procedure reported above (see chapter 4.2.1). Laboratory analyses were carried out within five months from blood collection at the Centre d'Etudes Biologiques de Chizé. CNRS-ULR, (UMR 7372) of Villiers en Bois, France.

5.2.2 Laboratory analyses

Telomere analysis was carried out using red blood cells as tissue samples. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol, and DNA concentrations and purity were checked using a spectrophotometer (Nanodrop ND-1000; Thermo Scientific, USA), according to previous recommendations (Nussey et al., 2014). Relative telomere length was measured using real-time quantitative PCR on a BioRad CFX 96 (Bio-Rad Laboratories, Hercules, California, USA), strictly following the protocol recently validated for wild vertebrates by McLennan and colleagues (McLennan et al., 2019).

5.2.3 Data analyses

All statistical analyses were run using R (version 3.5.3; R Core Team 2019).

First, I performed Linear Mixed Models (lme4 package) with a repeated measures design to test whether the change of telomer length over time differed between islands. In each model, I entered island (Sardinia *vs.* Corsica), sampling period (beginning *vs.* end of the experiment) and their interaction as fixed factors. I included individual identity as a random factor to control for non-independence of measurements. Preliminary models showed that entering sampling location within each island as a random factor did not improve the fitting of the model (i.e., the Akaike Information Criterion value was not reduced beyond 2), thus it was no longer considered.

Finally, I also tested whether there was any selective disappearance between the first and second sampling period of individuals from a given island having different values of telomere length compared to the overall distribution. I ran a Generalized Linear Model (glm in package 'lme4') with a binomial error distribution and a logit link function that included telomere length as main factor and survival probability (0 = dead, 1 = alive) until the end of the experiment as response variable.

5.3 Results

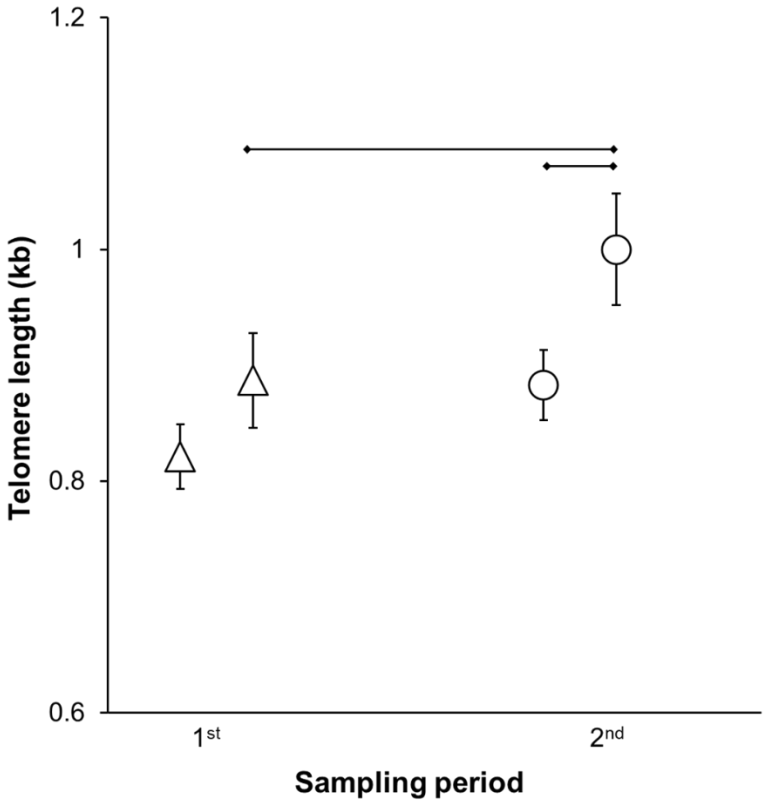
I found a significant effect of island, sampling period as well as of their interaction on telomere length variation (Table 1).

Table 1 Summary of LMMs. Telomere length was entered as dependent variables; island, sampling period and their interaction were entered as fixed factors; individual identity was included as a random factor. Significant factors are shown in bold.

Variable	Effect	F-value	p-value
Telomere length	island	7.07	**0.011**
	sampling	18.36	**<0.001**
	island x sampling	6.79	**0.013**

Post-hoc analyses by Tukey test show that tree frogs from both Corsica and Sardinia had similar telomere length at the beginning of the experiment (p-value=0.894). Conversely, at the end of the experiment, tree frogs from Corsica had longer telomers than those from Sardinia (p-value=0.005). Telomere length did not change significantly over the experiment in Sardinia tree frogs (p-value=0.357), while it increased significantly from the beginning to the end of the experiment in Corsica tree frogs (p-value=0.002). The changing in the telomere length for both sampling period are reported as EMMs ± SE from LMMs in Figure 1.

Figure 1 EMMs and standard errors from LMMs of the telomere length change over time between Sardinia (triangle) and Corsica (circle). Significant post-hoc (Tukey) contrasts are indicated with a bar.



Telomere length at the beginning of the experiment did not predict the probability to survive until the end of the experiment (p-value=0.959), indicating that there was no selective bias owing to tree frogs with shorter telomers having lower chances of survival.

5.4 Discussion

In this study, I investigated whether core (hereafter Sardinia) and edge (hereafter Corsica) populations of *Hyla sarda* differed in the telomere length as a consequence of a past range expansion. Using a longitudinal common garden experiment, I provide evidence of a significant differentiation in the telomere length among co-specific populations of tree frogs distributed along an historical route of range expansion from Sardinia to Corsica. To the best of my knowledge, this is the first time that co-specific populations showed striking differences in telomere dynamics. This result highlights that the species might not always be the appropriate unit of analysis in the telomere dynamics investigation.

At the beginning of the experiment, Sardinia and Corsica frogs showed similar telomere length, which means that the telomere length was not affected by the different ecological conditions occurred in the sampling areas. In contrast, at the end of the experiment, (i.e., one year later) Sardinia and Corsica frogs showed remarkable differences in their telomere length. Specifically, frogs from Corsica had had longer telomers than those from Sardinia. In fact, telomere length did not change significantly in Sardinia tree frogs, while it increased significantly from the beginning to the end of the experiment in Corsica tree frogs. These results were robust for any selective mortality that occurred during the experiment. Importantly, our longitudinal data covered a non-negligible portion of the expected lifespan of an individual (one out of approximately three years, Cadeddu et al., 2012), so that telomere attrition was a plausible expectation. Instead, no differences were observed in the Sardinia population while, a substantial lengthening of telomers occurred in the Corsica population. As pointed out above, Corsica population was founded during a recent range expansion from Sardinia. So, the evolution of these differences in telomere dynamics between the two populations should have occurred during the range expansion process or later.

An explanation for these results might lie with both adaptive and non-adaptive processes during range expansions or during subsequent isolation of populations. However, the absence of remarkable differences in the levels of genetic variability coupled with the lack of a genetic structure between Sardinia and Corsica populations (Bisconti et al., 2011a, b), suggest that the observed differences cannot be the result of no-adaptive processes (founder event and /or genetic drift). Moreover, the sampling design (see chapter 3.2.1), allow me to exclude major effects associated to different environmental conditions as probable drivers of the observed divergence in telomere dynamics. Hence, adaptive processes occurred during the range expansion event appear the most likely explanation for the evolution of the observed geographic pattern in telomere dynamics.

In vertebrates, telomere length is maintained and restored by the enzyme telomerase (Monaghan and Haussmann, 2006). Telomerase activity appears to be particularly relevant for the regulation of telomere dynamics in ectotherms (Olsson et al., 2018). Telomere elongation in Corsica but not Sardinia under common garden conditions suggests differential expression (i.e., re-activation or upregulation) of the telomerase in Corsica in response to these novel conditions. This suggest that the range expansion event may have promoted plasticity in telomere dynamics. Although my results provide first evidence of a spatial dimension within species in telomere dynamics, further studies will be needed to elucidate if such increased plasticity is an adaptation to the unpredictability of the novel environments, or to the non-equilibrium demographic dynamics encountered during the expansion process.

CHAPTER 6 GENERAL DISCUSSION

6.1 Discussion

Dispersal is an ecological process that shapes the distribution and evolution of phenotypes and genotypes across environments in a large number of species (Travis and Dytham, 2002; Phillips et al., 2010; Shine et al., 2011; Canestrelli et al., 2016a). A substantial evolution in dispersal-enhancing traits has indeed been extensively documented in populations undergoing range expansion, at least in successfully invader species (Phillips et al., 2006; Cote et al., 2010b; Chapple et al., 2012; Liebl and Martin, 2012; Brown et al., 2015; Courant et al., 2017; Gruber et al., 2017a; Kosmala et al., 2017). Although studies of ongoing invasion provide an excellent opportunity to investigate the causes and consequences of dispersal dynamics at work during range expansion, they do not allow the investigation of their biogeographic and evolutionary outcomes (Canestrelli et al., 2016b). In fact, the selective advantages associated with adaptive dispersal-enhancing traits during an expansion phase are transient as suggested by several empirical evidence (Travis and Dytham, 2002; Brown et al., 2007; Phillips et al., 2008; Brown et al., 2014) and we cannot say anything on their persistence in the populations. The recently established population may indeed evolve towards a condition of demographic equilibrium by recovering variation (phenotypic and genotypic) through a slower flow of individuals from the back (Cobben et al., 2015; Canestrelli et al., 2016a). To date, no studies have investigated if the directional changes in dispersal-enhancing traits persist in the populations. Looking at historical expansions is the only way to investigate consequences of dispersal processes and so far, no study yet has tackled this challenge.

This research tried to fill this gap by investigating if, in the context of Late Quaternary expansions, dispersal processes may have contributed to shape the geographic patterns in species phenotypic variation. To this end, the geographical variation of fourcategories of traits

(behaviour, locomotor performance,stress physiology and telomere dynamics) potentially implicated in dispersal processes (Dingemanse et al., 2003; Gruber et al., 2017a; Phillips et al., 2006; Louppe et al., 2017; Liebl and Martin, 2013; Selechnik et al., 2017), was characterized along the inferred route of the historical expansion of the tree frog species *Hyla sarda* (Bisconti et al., 2011b).

The results of this thesis show a geographic pattern of differentiation in all the investigated traits, which seems to retrace the route of past expansion thus suggesting that these traits have been possibly subject to important selective pressures during the *H. sarda* historical expansion. Moreover, I provided evidence of a personality in the *H. sarda*, a key factor for the evolution of virtually all feature of an organism (Réale et al., 2010; Wolf and Weissing, 2012; Canestrelli at al., 2016b), including dispersal propensity (Cote 2010a, b; Kelleher et al., 2018). In fact, in amphibians, emerging evidence suggests that personality could influence biogeographic processes, including island colonization (Brodin et al., 2013) and range expansions (Gruber et al., 2017a).

6.1.1 Phenotypic differentiation along a Pleistocene range expansion

To test a phenotypic differentiation as a consequence of a Pleistocene range expansion I compared tree frogs inhabiting Sardinia, the source area, with those that have colonized Corsica. The results demonstrate considerable phenotypic differentiation between Sardinia and Corsica populations in personality, locomotory performance, physiology as well as in telomere dynamics. Overall, Corsica individuals showed a shy personality coupled with a prudent exploration strategy; they had a greater jumping force at take-off, as well as higher adhesion force as compared to those from Sardinia. Corsica tree frogs displayed an exhaustion of antioxidant defenses (both GPx and OXY) coupled with a rise in oxidative damage (ROMs) and, a higher mortality rate as compared to their ancestors from Sardinia. Moreover, Corsica tree frogs showed a substantial lengthening of telomeres in respect to Sardinia. Such differences

suggest that behavioural, locomotory and physiological traits, as key components of dispersal propensity (Dingemanse et al., 2003; Gruber et al., 2017aa; Phillips et al., 2006; Louppe et al., 2017; Liebl and Martin, 2013; Selechnik et al., 2017, see introduction), were under strong selection during the past expansion.

The absence of remarkable differences in the levels of genetic variability coupled with the lack of a genetic structure between Sardinia and Corsica populations (Bisconti et al., 2011b), suggests that the observed differences cannot be the result of random processes (founder event and /or genetic drift), but instead of a deterministic process. Moreover, I can exclude a loss of genetic diversity caused by a possible reduction of suitable habitat. In fact, thanks to the increase of coastal lowlands (Van Andel and Shackleton, 1982; Shackleton et al., 1984) the species underwent a range expansion during Pleistocene ice ages (Bisconti et al., 2011a, b). The significant phenotypic differentiation assorted along the past south-north expansion axis makes the hypothesis of a deterministic process even more plausible. This hypothesis is in line with several theoretical and empirical studies of non-random dispersal (Fraser et al., 2001; Dingemanse et al., 2003; Cote and Clobert, 2007; Cote et al., 2010a; Canestrelli et al., 2016a). In fact, rapid changes in the phenotypic and genotypic makeup of populations undergoing range expansion have been frequently observed (Travis and Dytham, 2002; Simmons and Thomas, 2004; Phillips et al., 2006, 2010; Cote et al., 2010b; Shine et al., 2011; Chapple et al., 2012; Liebl and Martin, 2012; Brown et al., 2015; Canestrelli et al., 2016b; Courant et al., 2017; Gruber et al., 2017a; Kosmala et al., 2017).

On the whole, the emerging picture outlines a personality profile in sharp contrast with the combination of personality traits in a non-random expansion process. Indeed, dispersers at the range-edge have frequently been found bolder and more exploratory than those living in long established areas (Pintor et al., 2008; Liebl and Martin, 2012; Atwell et al., 2012; Myles-Gonzalez et al., 2015; Gruber et al., 2017a). Conversely, dispersers at the range-edge (Corsica) are shyer and characterized by a prudent exploratory strategy than those living in the ancestral

areas (Sardinia). The marked contrast of my results with those reported in literature (Pintor et al., 2008; Atwell et al., 2012; Liebl and Martin, 2012; Myles-Gonzalez et al., 2015; Gruber et al., 2017a) could be related to the species' ecology. It could be plausible that in a cryptic species, those individuals that successfully arrived in Corsica could have gained advantage by having a prudent exploration strategy reflecting a shy instead of a bold personality. A shy personality, indeed, could allow a more careful assessment of surrounding with a significant reduction of risks, ultimately increasing the chance of survival in a new environment. In Corsica rather than moving continuously tree frogs would minimize the activity rate, carefully evaluating the surrounding before to rapidly jump towards a new environment. Notably, the higher propensity to jump showed by tree frogs from Corsica is reflected in their better locomotory performance compared to tree frogs from Sardinia. As key traits in determining species dispersal ability, locomotory performance traits are directly implicated in dispersal processes and ultimately in expansion success. My results are in line with previous study on the locomotory performance traits in expanding populations (Phillips et al., 2008; Alford et al., 2009; Llewellyn et al., 2010) indicating that in the course of an expansion individuals may be spatially sorted by their dispersal ability (Phillips, Brown and Shine, 2010; Shine et al., 2011).

Overall, my findings depict a shy, prudent and performing descendant with a profile possibly constrained by the age-related oxidative status. In Corsica, an exhaustion of antioxidant defences coupled with a rise in oxidative damage was observed as respect to Sardinia. Moreover, Corsica frogs suffered higher mortality than those from Sardinia over the course of the experiment. Differences in the oxidative status between the two islands could be related to the differential way that distinct personalities cope with stress. An important body of literature indicates that shy personalities, across vertebrate *taxa*, respond to stress with a strong hypothalamic-pituitary-adrenal axis (HPA) stimulation and a consequent increase in circulating glucocorticoids compared to bold personalities (review in Carere et al., 2010). To cope with any stressful situations, vertebrates release glucocorticoids (Sapolsky et al., 2000; Romero,

2004) that may induce oxidative stress depending on exposure time (Costantini et al., 2011). In the expanding populations of house sparrow (*Passer domesticus*) a differential reactivity of the HPA-axis was observed, with individuals from range-edge releasing more corticosterone (Liebl and Martin, 2012, 2013). Corticosterone has long also been considered a promotor of dispersal by stimulating activity in birds and, recently, in movement-related survival in cane toads (Belthoff and Dufty, 1998; Jessop et al., 2018). Thus, Sardinia and Corsica frogs might have differed in the activity of the hypothalamic-pituitary-interrenal axis that releases corticosterone (Sapolsky et al., 2000; Romero, 2004). Here, I did not collect any glucocorticoids data, leaving opened the question of whether the corticosterone may be a pacemaker of age-related changes of oxidative status in tree frogs.

Notably, Rollins et al. (2015) found a substantial upregulation of genes involved in metabolism and cellular repair, including mechanisms of response to oxidative stress, in cane toad (*Rhinella marina*) at the edge of invasion front. These results suggested that dispersing cane toads might have prioritized investment in dispersal capacity and rapid demographic expansion rather than in self-maintenance mechanisms (Rollins et al., 2015). Analogously, natural selection might have favoured tree frogs that invested more into mechanisms that favour dispersal and colonisation, and this strategy came at a cost for the soma (higher oxidative stress and mortality) of Corsica frogs in the long-term. Thus, the high oxidative stress and mortality rate found in Corsica tree frogs may represent an imprint of physiological cost to maintain dispersal traits useful to successfully colonize the northern portion of their range. This hypothesis seems supported by the findings that changes in oxidative status emerged only after one year in common garden conditions. Moreover, this would suggest that the differences in the oxidative status were not environmental-driven, but rather constrained by endogenous metabolic mechanisms that perhaps distinguish the populations of the two islands.

Similarly, the remarkable differences in the telomere length between Sardinia and Corsica emerged over time (after one year in common garden conditions). Specifically, Corsica frogs

showed a substantial lengthening of telomers in respect to Sardinia suggesting a differential expression of the telomerase in Corsica in response to the common garden conditions. Previous population genetic data (Bisconti et al., 2011a, b), together with the sampling design (see chapter 3.2.1), make adaptive processes occurred during the range expansion the most likely explanation for the evolution of this geographic pattern in telomere dynamics.

Although my results provide first evidence of a spatial dimension within species in telomere dynamics, they suggest the intriguing scenario that non-equilibrium processes, such as range expansions, might promote plasticity in the molecular machinery regulating telomere dynamics.

6.1.2 Phenotypic differentiation shaped by Pleistocene island colonization

To test a phenotypic differentiation as a consequence of a Pleistocene island colonization I compared tree frogs inhabiting Corsica with those that have colonized the Elba island. The results of my work show a substantial differentiation in behavioural and locomotory traits across populations inhabiting these long been separated islands. Overall, individuals from Elba were bolder but less exploratory, they had a worse jumping force at take-off, as well as lower adhesion force than their ancestors from Corsica. Unfortunately, tree frogs from Elba island had to be excluded by the oxidative status (see chapter 5.2) as well as by the telomere dynamics characterization (see chapter 6.2).

The Elba frogs' low genetic diversity, as compared to the rest of the species' range, as well as its geographic location at the northern edge of the species' range (Bisconti et al., 2011b), suggests that the observed differences could be generated by a random founder event. A past founder event could have contributed to fix such traits, explaining why such phenotypic differences would persist over time. Moreover, the isolation may have precluded the recruitment of other alleles, preventing the evolution of such traits over time. In fact, the role of insularity in producing phenotypic differences among populations has long been recognized

(Wallace, 1880; Foster, 1964; MacArthur and Wilson, 1967; Blumstein, 2002; Lomolino, 2005; Whittaker and Fernández–Palacios, 2007; Forsman et al., 2011).

Alternatively, it might be that the phenotypic make-up, that probably could have helped colonizers to reach the Elba island, may not have favoured later generations to survive in isolated conditions. It is possible that the overseas dispersal events (Vences et al., 2003, 2004), a very hostile and risky matrix to amphibians, have strongly selected only few successful disperser phenotypes able to reach the Elba island. This hypothesis is in agreement with growing evidence that dispersers, in this case founders, carry a suite of dispersal-enhancing traits, including personality and locomotory traits (O'Rian et al., 1996; Phillips et al., 2006, 2008; Cote et al., 2010b; Louppe et al., 2017; Gruber et al., 2017a). The current personality differences may have been generated by a mechanism of adaptive differentiation to the island ecological release, the so-called 'Island Syndrome', (e.g. rodents: Adler and Levins, 1994; birds: Blondel, 2000; reptiles: Novosolov et al., 2013). The boldness may have been maintained over time by the absence of a counter selective force in a relaxed ecological context. Several studies indicate that predation, typically reduced on islands (MacArthur and Wilson, 1967; Blumstein and Daniel, 2005; Cooper, Pyron and Garland, 2014), is one of the most important forces in shaping the phenotype including personality traits (Bell et al., 2005; Dingemanse et al., 2007; Bell and Sih, 2007). It could be that a relaxation in selective pressure owing to predation may have caused a shift in the behavioural repertoire of the founding population leading to an increase in the expression of bold phenotypes, as well as a decrease in explorative phenotypes. Examples of bold personality in low predation pressure environments are numerous (Lima and Dill, 1990; Anholt and Werner, 1998; Brodin and Johansson, 2004; Bell and Sih, 2007).

Another factor, which is typical of islands, is reduced food availability: islands tend to have both lower abundance and diversity of arthropods, which are the main food source for many reptiles and amphibians (Janzen, 1973; Olesen and Valido, 2003) and it is possible that a bold

personality could be more successful in an environment with a limited access to resources (De Meester et al., 2018). On the other hand, exploratory abilities of the founding population could be lost over time because not beneficial in a limited environment. However, in absence of data on the predation pressure and prey availability, I can only hypothesise that these factors may have contributed to the observed patterns.

The strong reduction in dispersal potential on islands has been observed in many diverse *taxa* (Cody and Mc Overton, 1996 and reference therein). It is possible that costly locomotory performance may have been counter-selected by the ecological relaxation, thus determining the observed differences (Blumstein et al., 2002; Blumstein et al., 2005). To date, empirical evidence for the effect of insularity on personality and performance traits are scanty and frequently not accompanied by inferred demographic history (Cody and McC Overton, 1996 and reference therein; Forsmann et al., 2010) preventing any comparisons with my results. Although, further studies are needed to understand which processes may have been involved in shaping the phenotypic profiles on Elba island, I provide a first evidence of the crucial role of past biogeographic processes in determining the patterns of island populations phenotypic variation.

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APPENDIX

1. Biogeographic history moulds population differentiation in physiological ageing in an amphibian

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Abstract

Regulation of oxidative status plays a substantial role in physiological ageing. However, we know little about age-related changes of oxidative status in wild animals, and even less about the role of population history in moulding ageing rates. We addressed these questions by means of a common garden experiment, using the Tyrrhenian tree frog Hyla sarda as study species. This species underwent a range expansion from northern Sardinia (source) up to Corsica (newly founded) during the Late Pleistocene, and then the two populations became geographically isolated. We found that, at the beginning of the experiment, Sardinia and Corsica frogs had similar concentrations of all oxidative status markers analysed. One year later, Corsica frogs had higher oxidative stress and suffered higher mortality than Sardinia frogs. Our results suggest the intriguing scenario that population differentiation in rates of physiological ageing owing to oxidative stress might be an overlooked legacy of past biogeographic processes.

Keywords: Antioxidant, Oxidative stress, Range expansion, Senescence, Vertebrates.

Introduction

Ageing is a ubiquitous process of decline in physiological function, survival and fecundity with advancing age (de Magalhães and Passos, 2018). In recent times there has been growing interest in elucidating the physiological mechanisms of ageing in free-living animals or non-model laboratory organisms (Gaillard and Lemaître, 2020). The oxidative stress theory of ageing postulates that age-associated reductions in physiologic functions are caused by slow steady changes of oxidative status with age (e.g., accumulation of molecular oxidative damage), which that are associated with life expectancy of organisms (Finkel and Holbrook, 2000; Partridge and Gems, 2002). However, we still know relatively little about age-related changes in oxidative status markers within individuals in wild animals. This research gap is particularly unfortunate if we consider the bourgeoning interest in understanding the functional and fitness consequences of within- and among-individual variation in oxidative status (Costantini, 2014). A pillar of this research is that the expression of oxidative status traits might integrate the individual history or genetic background and underlie life-history trade-offs (Costantini, 2014). For example, variation of oxidative status markers may be linked to immune function (Sorci and Faivre, 2009), the expression of secondary-sexual traits (Alonso-Alvarez and Galvan, 2011), personality traits (Herborn et al. 2011), chronic stress exposure (Hau et al., 2015) or demographic traits (Costantini et al., 2015). This emerging view of oxidative status biology as an integrative research ground, suggests potential implications of physiological ageing in eco-evolutionary processes that mould the integrative phenotypes, such as dispersal.

Dispersal is a key context-dependent process that contributes to shape spatial patterns of biodiversity at all levels of organization, from genes to ecosystems (Hanski and Gilpin, 1991; Hansson, 1991; Bohonak, 1999; Ronce, 2007; Clobert et al., 2012; Saastamoinen et al., 2018). Recent theoretical and empirical evidences, however, suggest that dispersal could also depend on individual state or phenotypic quality, which can either influence or be influenced by the

evolution of virtually all the features of an organism, including behaviour, morphology, physiology, and genetics (Clobert et al., 2012, Canestrelli et al., 2016a, b). Dispersal is a highly energetically and resource demanding process that imposes a number of physiological challenges (Bonte et al., 2012).

Importantly, dispersal out of the range-edge of an expanding population can impose ecological pressures to dispersers, resulting from novel biotic interactions (Sakai et al., 2001; Lee and Klasing, 2004; Brown et al., 2013) or abiotic conditions (e.g., temperature, humidity) at which they need to physiologically adapt (Wilson et al., 2000; Johnston and Temple, 2002; Steinhausen et al., 2008). Several lines of evidence showed that metabolic activity (Myles-Gonzalez et al., 2015; Louppe et al., 2018) or the immune function (White and Perkins, 2012; Cornet et al., 2016) may differ between edge and core populations. This is particularly relevant because both metabolic rate and immune function may affect certain traits of oxidative status (Costantini, 2014), strengthening the idea that oxidative status and its role in physiological ageing might vary among populations across an expansion gradient.

In this study, we have tested for the first time whether dispersal-driven processes of historical biogeographic relevance, such as range expansions, have moulded physiological ageing rates by leaving detectable imprints of this influence on age-related changes in oxidative status markers. We hypothesize that, if individual variation in physiological ageing reflects the variation in the capacity to cope with the new demographic and/or environmental conditions encountered during a range expansion, a spatial structure in oxidative status might emerge along the range of a recently expanded population. We have explored this hypothesis by means of a longitudinal common garden experiment lasting one year, using the Tyrrhenian tree frog *Hyla sarda* as study species. The Tyrrhenian tree frog underwent a range expansion from northern Sardinia (core) up to Corsica (edge) during the Late Pleistocene and the expansion was promoted by the opening of a wide land bridge between the two islands (Bisconti et al., 2011a, b). Specifically, we have studied the spatial differentiation along the inferred route of the

species' past expansion in four blood-based markers of oxidative status, including damage level and both non-enzymatic and enzymatic antioxidants.

Results

We found a significant interaction between island and sampling period for ROMs, OXY, GPx and SOD/GPx ratio (Table 1). Post-hoc analyses by Tukey test showed that islands had similar values of all tested markers at the beginning of the experiment (Figure 1). Conversely, at the end of the experiment, Corsica tree frogs had significantly higher ROMs ($p = 0.013$) and SOD/GPx ratio values ($p = 0.022$), and lower OXY ($p = 0.005$) and GPx ($p = 0.038$) than those from Sardinia (Figure 1). From the beginning to the end of the experiment, ROMs decreased in Sardinia ($p = 0.003$) and increased in Corsica ($p = 0.028$); GPx increased in Sardinia ($p = 0.007$) and decreased in Corsica ($p=0.015$); OXY did not change in Sardinia and decreased in Corsica ($p < 0.001$). SOD increased significantly in both Sardinia ($p = 0.006$) and Corsica ($p = 0.038$) over the course of the experiment; the SOD/GPx ratio increased significantly in Corsica frogs ($p = 0.001$) (Figure 1). All markers of oxidative status were significantly repeatable over the course of the experiment; estimates of repeatability coefficients varied from 0.25 for OXY to 0.48 for GPx (Table 2).

We recorded a higher mortality of frogs from Corsica (8 out of 23, 34.8%) than those from Sardinia (1 out of 32, 3.1%; $p = 0.002$). Values of each marker at the first sampling of blood did not predict the mortality probability until the second sampling of blood in frogs from Corsica (p-values ≥ 0.41), indicating that results were robust for any potential bias introduced by selective mortality.

Discussion

Using a longitudinal common garden experiment, we show compelling evidence of a significant differentiation in age-related changes of oxidative status markers between populations of tree frogs along an historical range expansion route from Sardinia (core population) to Corsica (edge population). At the beginning of the experiment, we found that Sardinia and Corsica frogs had similar concentrations of all oxidative status markers. One year later (i.e., at the end of the experiment), Sardinia and Corsica frogs had sharp differences in most markers analysed. Specifically, frogs from Corsica had significantly higher ROMs (marker of oxidative damage) and of SOD/GPx ratio values (higher values indicate faster cellular ageing; De Haan et al., 1995, 1996), and lower antioxidant molecules (OXY and GPx) than those from Sardinia. In contrast, we found that expression of the antioxidant enzyme SOD was increased at the end of the experiment in both islands. Importantly, we also found that frogs from Corsica suffered higher mortality than frogs from Sardinia over the course of the experiment. Overall, these results point to a differentiation in the pace of physiological ageing between conspecific populations that have been geographically and genetically isolated since the end of the Pleistocene epoch (Bisconti et al., 2011a). Our results were robust for any selective mortality that occurred during the experiment and for environmental variation because frogs were maintained under the same common garden conditions.

Mitochondrial activity is one important source of the free radical superoxide anion that, in turn, is precursor of other pro-oxidants, such as hydrogen peroxide (Halliwell and Gutteridge, 2015). SOD, an enzyme that protects against the superoxide anion, was upregulated over the course of the experiment in both islands. Thus, we might exclude a strong role of mitochondrial superoxide anion in explaining our results if the level of SOD expressed in the two islands was similarly effective in controlling pro-oxidant activity of superoxide anion. The upregulation of SOD with time is consistent with prior longitudinal and cross-sectional studies on captive zebra

finches (Marasco et al., 2017) and free-ranging cheetahs (Costantini et al., 2017), indicating that exposure to certain free radicals might increase with age as shown by research on laboratory strains (Costantini, 2014).

Alternatively, Sardinia and Corsica frogs might have differed in the activity of the hypothalamic-pituitary-interrenal axis that releases corticosterone (Sapolsky et al., 2000; Romero, 2004). Corticosterone promotes a suite of metabolic processes that increase gluconeogenesis, catabolism of energy reserves, inhibition of energy storage, and increase in locomotor and foraging activity (Sapolsky et al., 2000). Corticosterone can also induce changes in oxidative status, especially when animals are being exposed to increased levels of corticosterone for long periods of time (Costantini et al., 2011). In the house sparrow (*Passer domesticus*), individuals from range-edge produced more corticosterone, possibly in response to their exposure to novel environmental conditions (Liebl and Martin, 2013; Martin and Liebl, 2014). Corticosterone has long also been considered a promotor of dispersal by stimulating activity in birds and, recently, in movement-related survival in cane toads (Belthoff and Dufty, 1998; Jessop et al., 2018). Further work will be needed to elucidate the role of corticosterone as a pacemaker of age-related changes of oxidative status in tree frogs.

The results of our work raise the important question of why these population differences in oxidative status have evolved. An explanation might lie with the legacy of a past positive selection on those life-history strategies that were more successful during the range expansion. Rollins et al. (2015) found a substantial upregulation of genes involved in metabolism and cellular repair, including mechanisms of response to oxidative stress, in cane toads at the edge of invasion front. These results suggested that dispersing cane toads might have prioritized investment in dispersal capacity and rapid demographic expansion rather than in self-maintenance mechanisms (Rollins et al., 2015). This strategy would be successful whether the costs of reducing protection against oxidative damages are low as compared to those incurred to sustain active defense mechanisms. This explanation is congruent with the disposable soma

hypothesis of ageing, which states that optimisation of trade-offs in the allocation of limited resources among self-maintenance and other activities inevitably comes at a cost for the soma in the long-term (Kirkwood 1977; Kirkwood and Rose 1991). Similarly, the antagonistic pleiotropy hypothesis of ageing posits that natural selection would favour the evolution of life histories in which alleles have beneficial effects on fitness early in life even when they are detrimental in old individuals (Williams, 1957; Nussey et al., 2013). The cost of ageing would be low because it would emerge at a phase of lifetime (old age) when the force of natural selection is expected to be low. Our longitudinal data actually covered a non-negligible portion of the expected lifespan of a treefrog (one out of approximately three years; Cadeddu et al., 2012). The selection pressures produced by extrinsic mortality factors are thought to play a major role in the evolution of ageing rates (Monaghan et al., 2008). The risk of death from extrinsic causes, such as disease or predation, during dispersal is particularly important (Clobert et al., 2012). Investing resources in a robust and long-lasting body might be maladaptive if extrinsic causes of mortality are high. In these circumstances a much better strategy, in evolutionary terms, would be to invest more resources into traits that favour dispersal. Thus, natural selection might have favoured frogs that invested more into mechanisms that favour dispersal and colonisation, and this strategy came at a cost for the soma (higher oxidative stress and mortality) of Corsica frogs in the long-term.

Finally, we found a moderate individual repeatability of all measured markers (0.25 to 0.48). Estimates of within-individual repeatability of oxidative status markers are rarely reported in the literature and the few available are in line with our estimates (e.g., Saino et al., 2011; Récapet et al., 2019). The mechanistic reasons for and biological meaning of these intrinsic individual differences in oxidative status markers have not been elucidated yet. We argue that, together with the clear spatial structure we found, this significant consistency suggests that the pace of physiological ageing might be heritable and that selection on oxidative status might contribute to drive the evolution of individual reaction norms.

In conclusion, our experiment provides strong evidence for a role of adaptive processes that occurred during an historical range expansion in explaining the different pace of physiological ageing between populations of tree frogs. These results also indicate that, while age-related changes in SOD might be a shared (or conserved) mechanism (*sensu* Partridge and Gems 2002) of ageing, those in other oxidative status markers (ROMs, serum non-enzymatic antioxidants and GPx) might be private (peculiar to particular evolutionary lineages) because they were expressed only in Corsica frogs. The possible conserved SOD expression across the two islands is also supported by the lack of genetic variation between Sardinia and Corsica frogs in the gene SOD-1 (Bisconti et al., 2011b). Further studies will be needed to elucidate the endogenous mechanisms responsible for the observed differences in oxidative status and the correlated functional and fitness consequences. It will also be important to determine whether such differences are owing to genetic variants or developmental plasticity.

Material and Methods

Sampling and housing

A total of 55 males of *H. sarda* was included in this study. Animals were caught along a latitudinal transect (Figure 2) that goes from the putative area of glacial refuge in central-eastern Sardinia up to the northern Corsica (Bisconti et al., 2011a). All sampling locations were in coastal areas which, following an assessment of current and peri-glacial bioclimatic suitability, resulted substantially homogeneous, both in space and time (Bisconti et al., 2011a). This sampling scheme allowed us to minimize possible bioclimatic effects affecting the geographic patterns of the investigated markers.

Male tree frogs were collected with hand nets following mating calls at night in early summer, transported into our laboratory and housed in individual cages (25 cm × 25 cm × 25 cm) in a climatically controlled room at a temperature of 24/25°C, relative humidity of 60-80%, and natural photoperiod. Cages were provided with a dechlorinated water tank, a plant, an oak wood as shelter, and were placed randomly with respect to population origin. Twice a week cages were cleaned, water was renewed, and tree frogs were fed with crickets (*Acheta domestica*).

Ethical note

Sampling procedures were performed under the approval of the Institute for Environmental Protection and Research 'ISPRA' (protocol # 5944), Ministry of Environment 'MATTM' (protocol #8275), Regione Sardegna (#12144) and Corsica (#2A20180206002 and #2B20180206001). Permission to temporarily house amphibians at CISMAR was granted by A.S.L. Tarquinia (Local Health and Veterinary Centre) with license code 050VT427. All handling procedures outlined in the present study were approved by the Ethical Committee of the Tuscia University for the use of live animals. During captivity the animals were monitored

daily. No adverse effects on the overall health of tree frogs were observed during the procedures. The animals were released in the original sampling locations at the end of the experimentation.

Blood sampling

Blood samples were collected by cardiac puncture with a 0.3 ml syringe from 55 individuals (32 from Sardinia and 23 from Corsica). One year later, another sample of blood was collected from 46 individuals still alive (31 from Sardinia, 15 from Corsica). Before the collection of blood, animals were anesthetized by immersion for two minutes in a solution of MS-222 (Tricaine methanesulfonate, 0.5% m/v). Blood samples were straightaway centrifuged at 10,000 rpm for 3 minutes in order to separate the serum from the erythrocytes in two different tubes that were immediately stored at -80°C until laboratory analyses. After blood collection, each individual was placed in a wet box; we returned frogs to their cages when they were fully awakened.

Laboratory analyses

Four markers of oxidative status were measured according to established protocols for vertebrates (Costantini et al., 2011). Briefly, we used the d-ROMs test (Diacron International, Grosseto, Italy) to quantify oxidative damage in serum. The ROMs concentration is expressed as mM of H_2O_2 equivalents. The OXY Adsorbent test (Diacron International, Grosseto, Italy) was used to quantify the ability of serum non-enzymatic antioxidant compounds (vitamins, carotenoids, uric acid, thiol proteins) to react *in vitro* with a known concentration of hypochlorous acid (HOCl). OXY values were expressed as mM of HOCl neutralised/mg proteins. The Ransel assay (Randox Laboratories, Crumlin, UK) was used to measure the activity of the antioxidant enzyme glutathione peroxidase (GPx) in hemolysates. The activity of GPx was expressed as Units/mg of proteins. The Ransod assay (Randox Laboratories,

Crumlin, UK) was used to quantify the activity of the antioxidant enzyme superoxide dismutase (SOD) in hemolysates. The activity of SOD was expressed as Units/mg of proteins. Concentration of proteins in hemolysates was quantified using the Bradford test. All the analyses were run in duplicate, and the mean coefficient of variation ranged between 6.88% and 8.82%.

Statistical analyses

Statistical analyses were run using R (version 3.5.3; R Core Team 2019). Generalized linear mixed models (lme4 package) with a repeated measures design were used to compare frogs from the two islands for each blood-based oxidative status marker. In each model, we included island (Sardinia and Corsica), sampling period (beginning and end of the experiment) and their interaction as fixed factors; individual was included as random factor to control for non-independence of repeated measurements. As dependent variables, we entered each marker singly; we also run a model using the SOD/GPx ratio as dependent variable because prior work suggested that an unbalanced ratio of the two enzymes (i.e., higher values) might be associated with cellular ageing (De Haan et al., 1995, 1996). A Gamma distribution and a log link function was used for ROMs, OXY and SOD/GPx, while a Gamma distribution and an identity link function was used for GPx and SOD.

Preliminary models showed that entering sampling location within each island as a random factor did not improve the fitting of the model (i.e., the Akaike Information Criterion value was not reduced beyond 2), thus it was no longer considered. Preliminary models also showed that the fitting of models based on the whole dataset was better than those of models based on the subset of individuals of which we had data for both the pre- and post-experimental period (data not shown).

Generalized linear mixed effect (GLMM)-based repeatability models (glmer in package ‘lme4’) were then used to quantify the within-individual repeatability of all oxidative status markers.

Gamma distribution with a log link function was used. The observation-level variance was obtained by using the trigamma function (Nakagawa et al, 2017). We entered each marker singly as a dependent variable and individual as random factor. Preliminary models showed that the adjusted repeatability calculated by entering location as fixed factor did not improve the fitting of the models.

Finally, we compared the mortality percentage observed from the first to the second sampling of blood between islands using [p-value = $(p_s \times n_s + p_c \times n_c)/(n_s + n_c)$] in STATISTICA Version 10 (StatSoft, Tulsa, OK, USA), where p is the proportion of dead frogs, n is the sample size, s is Sardinia and c is Corsica. We also tested whether there was any selective mortality between the first and second sampling period of individuals from a given island having different values of each marker at the beginning of the experiment as compared to the overall distribution. We run a generalized linear model (glm in package lme4) with a binomial error distribution and a logit link function that included a given marker as main factor and survival probability (0 = dead, 1 = alive) until the end of the experiment as response variable. These analyses were restricted to Corsica because only one frog from Sardinia died before the second sampling of blood.

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Author Contributions: Daniele Canestrelli, David Costantini, Roberta Bisconti and Claudio Carere designed research; David Costantini, Anita Liparoto, Daniele Canestrelli, Roberta Bisconti and Claudio Carere performed research; David Costantini and Anita Liparoto analyzed data and wrote the paper with inputs from the other authors.

Additional files

Supplementary files: Geographic coordinates of sampling locations and values of oxidative status markers, for each individual tree frog analysed in this study.

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Table 1. Outcomes of generalized linear mixed models. Markers were entered as dependent variables; island, sampling period and their interaction were entered as fixed factors; individual identity was included as a random factor. Significant factors are shown in bold.

Variable	Effect	F-value	p-value
ROMs	Island	1.05	0.630
	Sampling period	0.86	**0.028**
	Island × Sampling period	9.09	**0.001**
OXY	Island	2.99	0.745
	Sampling period	11.20	<**0.001**
	Island × Sampling period	6.11	**0.010**
GPx	Island	0.72	0.484
	Sampling period	0.28	**0.015**
	Island × Sampling period	8.35	**0.001**
SOD	Island	0.04	0.606
	Sampling period	11.32	**0.038**
	Island × Sampling period	0.002	0.954
SOD/GPx	Island	1.11	0.824
	Sampling period	4.46	**0.002**
	Island × Sampling period	6.12	**0.013**

Table 2. Outcomes of GLMM-based repeatability estimates. Markers were included in the models as dependent variable and the individual was entered as a random factor. Gamma distribution with log link function was used. The observation-level variance was obtained using the trigamma function in R.

Variable	Estimate	Observation-level variance
ROMs	0.40	0.017
OXY	0.25	0.061
GPx	0.48	0.139
SOD	0.26	0.160
SOD/GPx	0.36	0.216

Figure captions

Figure 1. Estimated marginal means and standard errors obtained from generalized linear mixed models for Sardinia and Corsica frogs. ROMs = reactive oxygen metabolites in serum; OXY = serum non-enzymatic antioxidant capacity; GPx = glutathione peroxidase; SOD = superoxide dismutase. Significant contrasts are indicated with bars. The study species *Hyla sarda* is shown in the lower right panel.

Figure 2. Geographical distribution of sampling locations (green shapes). Green arrow indicates the approximate route of late-Pleistocene range expansion of the Tyrrhenian tree frog [30,31]. Dashed line shows the coastline during the last glacial maximum (21,000 years ago).

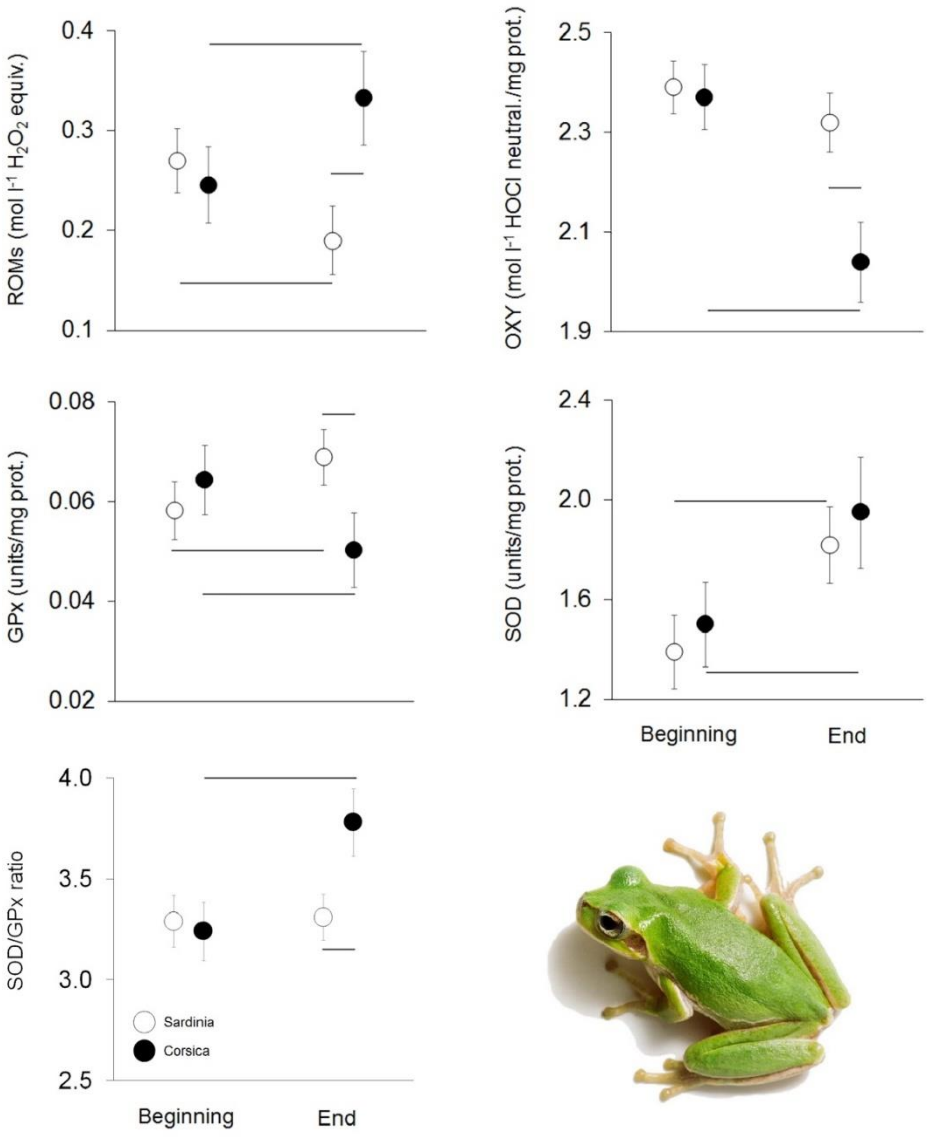
ROMs (mol l-1 H2O2 equiv.)
0.4
0.3
0.2
0.1
OXY (mol l-1 HOCl neutral./mg prot.)
2.5
2.3
2.1
1.9
GPx (units/mg prot.)
0.08
0.06
0.04
0.02
SOD (units/mg prot.)
2.4
2.0
1.6
1.2
Beginning
End
SOD/GPx ratio
4.0
3.5
3.0
2.5
Sardinia
Corsica
Beginning
End

Figure 1

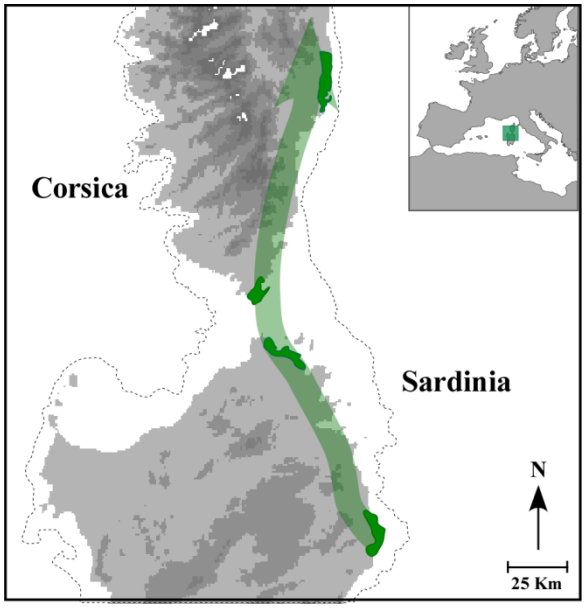

Figure 2

2. Biogeography of telomere dynamics in a vertebrate

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Abstract

Telomere length variation has been implicated in processes of ecological and evolutionary importance in a wide range of organisms. However, while the temporal component of this variation has been the subject of much research, we do not know yet whether a spatial component exists within species in telomere dynamics. Here, we investigated whether the biogeographic history of populations, a key driver of eco-evolutionary processes, can promote the evolution of telomere dynamics. Based on a one-year longitudinal common-garden experiment in a tree frog species we found, for the first time, that co-specific populations can show striking differences in telomere dynamics, not explained by distinct environmental conditions experienced by individuals through time. Indeed, the populations did not differ in telomere length at the beginning of the study, yet they did along the experiment. We observed stability of telomere length over time within historically stable populations, but remarkable elongation (31.2% on average) within populations arisen during a recent range expansion. Our results suggest the intriguing scenario that non-equilibrium processes, such as range expansions, might promote plasticity in the molecular machinery regulating telomere dynamics.

Introduction

Telomeres are nucleoprotein structures located at the terminal ends of chromosomes that play a major role in maintaining chromosome and genome stability and in avoiding loss of genetic material (1). In recent times, there has been a bourgeoning interest in understanding the fitness consequences of variation in telomere length and dynamics (2). A core idea is that telomere length might integrate the individual history, thus representing a valuable metric of individual phenotypic quality (3). For example, the telomere dynamics may be affected by habitat quality (4), food availability (5), intraspecific competition (6), immune function (7), chronic stress exposure (8), inbreeding (9), and have been associated with personality traits and the expression of sexual weapons (10, 11). This emerging integrative view of telomere biology, raises immediate questions about its potential implications in eco-evolutionary processes that are prototypical of the integrative phenotype, such as dispersal.

Dispersal is a key process in ecology and evolution, which contributes shaping spatial patterns of distribution of biological diversity at all levels of organization (12). It is implicated in a wide range of processes, from the spread of new genetic variants among populations, to range expansions into previously unoccupied geographic regions, to demographic and metapopulation dynamics, to community assembly and even ecosystem functioning (12-14). In a biogeographic perspective, dispersal has long been acknowledged as an inherently context-dependent process (15). More recently, however, dispersal has also emerged as an essentially condition-dependent process, which can influence - and be influenced by - the evolution of virtually all the features of an organism, including behaviour, morphology, physiology, and genetics (12, 16-19).

Here, we ask for the first time whether dispersal-driven processes of historical biogeographic relevance, such as range expansions, can affect telomere length and telomere dynamics and leave detectable imprints of this influence. We hypothesize that, if individual variation in

telomere dynamics translates into heritable variation in the capacity to cope with the new demographic and/or environmental conditions encountered during a range expansion, a spatial structure in telomere dynamics might emerge along the range of a recently expanded population. We explore this hypothesis using the Tyrrhenian tree frog *Hyla sarda* as study species. This is a small, cryptically coloured amphibian endemic to the Tyrrhenian islands, which colonized the northern portion of its current range during a Late Pleistocene range expansion (20, 21). This northward diffusion process was promoted by the temporary, glaciation-induced opening of a wide land bridge between the source area (northern Sardinia island) and the colonized area (Corsica island) (20, 21). Using a long-term longitudinal common garden experiment (12 months) during which animals have been housed under identical conditions, we compared telomere length and its change over time between individuals sampled in the source area in Sardinia and individuals sampled in the expansion range in Corsica.

Results

We found significant effects of geographic area ($F = 10.25$, $p = 0.002$), sampling time ($F = 21.59$, $p < 0.001$) and of their interaction ($F = 6.60$, $p = 0.014$). Post-hoc analyses showed that tree frogs from both Corsica and Sardinia had similar telomere length at the beginning of the experiment ($p=0.549$), while tree frogs from Corsica had longer telomeres than those from Sardinia at the end of the experiment (coeff. estimate ± SE: 0.218 ± 0.057, $p=0.002$) (Figure 1). Telomere length did not change significantly over the experiment in Sardinia tree frogs ($p=0.229$), while it increased significantly from the beginning to the end of the experiment in Corsica tree frogs (-0.215 ± 0.050, $p=0.0006$; 31.2% elongation on average) (Figure 1). All frogs included in this study survived until the end of the experiment, indicating that there was no selective bias owing to tree frogs with shorter telomeres having lower chances of survival.

Discussion

In recent years, there has been growing interest in understanding the biological meaning of telomere length and dynamics in a wide range of species (2). The species, however, might not be the appropriate unit of analysis. Indeed, our study showed, for the first time, that different co-specific populations can show striking differences in telomere dynamics, and that these differences cannot be explained by distinct environmental conditions experienced by individuals through time. We also did not observe any differences between populations in telomere length at the beginning of the study; rather these differences emerged along a longitudinal common-garden experiment, indicating that point estimates (e.g., as routinely used when comparing sexes or developmental stages) might provide blurred pictures of interindividual variation in telomere dynamics.

Telomeres shorten with age in most organisms studied to date (22), but not in the Tyrrhenian tree frog. Our longitudinal data covered a non-negligible portion of the expected lifespan of an individual tree frog (one out of approximately three years, 23), so that telomere attrition was a plausible expectation. Instead, we observed no differences between time points in the Sardinia population, and a substantial lengthening of telomeres in the Corsica population. Corsica population was founded during a recent range expansion from the Sardinia population. So, the evolution of the observed differences in telomere dynamics between the two populations should have occurred during the range expansion process or later.

Both adaptive and non-adaptive processes can promote phenotypic change during range expansions or during subsequent isolation of populations. However, previous population genetic data (20, 21), together with our sampling design, make both non-adaptive processes (genetic drift) and environmental adaptation following colonization, as improbable drivers of the observed divergence in telomere dynamics (see Methods). Hence, adaptive processes occurred during the range expansion event appear the most likely explanation for the evolution of this geographic pattern in telomere dynamics.

In vertebrates, telomere length is maintained and restored by the enzyme telomerase (24). Telomerase activity appears to be particularly relevant for the regulation of telomere dynamics in ectotherms (25). Telomere elongation in Corsica but not Sardinia under common garden conditions suggests differential expression (i.e., re-activation or upregulation) of the telomerase in Corsica in response to these novel conditions. This does imply that the range expansion event promoted plasticity in telomere dynamics, adding perspective to the burgeoning focus on the role of plasticity in evolution (26). Whether such increased plasticity is an adaptation to the unpredictability of the novel environments, or to the non-equilibrium demographic dynamics encountered during the expansion process, is a further intriguing subject for future research.

Materials and Methods

Sampling and common garden experiment

We sampled 47 Tyrrhenian tree frogs from the Sardinia and Corsica islands (western Mediterranean basin; see Fig. 1). Our sampling strategy was designed to control for potentially confounding factors, which may hamper subsequent data interpretation. First, we collected only adult tree frogs, since differences between life stages in telomere dynamics have been documented (27). Second, although the species can be found in a variety of altitudes and freshwater environments, we sampled only in coastal pools to avoid major effects associated to different environmental features (4, 5). Third, we sampled 2 geographic areas per island (Fig.1), 2 breeding sites per geographic area (i.e. 8 sites; mean distance ±s.d.= 58.8 ±38.3 km and 60.6 ±40.5 km, for Sardinia and Corsica island, respectively), and a minimum of 2 distinct pools within each site. This allowed us to control for potential effects of local habitat (see above) or demographic features (e.g. kinship, inbreeding; see 9, 28). Fourth, bioclimatic differences between geographic areas and across time epochs (current vs. last glacial maximum) were minimized, by limiting sampling activities to a narrow coastal strip at the eastern side of the species range. Indeed, in a previous species distribution modelling (20, 21), this side received comparatively higher bioclimatic suitability scores under both current and past bioclimatic conditions. Fifth, although the species is also present in the northern islands of Capraia and Elba, we deliberately left these islands unsampled, as they were probably colonized through jump dispersal (i.e. via founder events, with consequent genetic drift), rather than by a spatial diffusion process (20, 21).

Tree frogs were collected with hand nets following mating calls at night in early summer, and housed in individual cages (25 cm x 25 cm x 25 cm) within a climatically controlled room at a temperature of 24/25 C°, relative humidity of 60-80 %, and natural photoperiod. Cages were

provided with a dechlorinated water tank, a plant, an oak wood as shelter, and were placed randomly with respect to population origin. Twice a week cages were cleaned, water was renewed, and tree frogs were fed with crickets (*Acheta domestica*).

Sampling and experimental procedures were approved by the Italian Ministry of Environment 'MATTM' (protocol #8275), and Prefecture of Corsica (#2A20180206002 and #2B20180206001).

Telomere analyses

Telomere analysis was carried out using red blood cells as tissue samples. Blood samples were collected twice: two weeks after tree frogs arrived in lab, and exactly one year later.

Tree frogs were anesthetized by submersion in a solution of MS-222 (0.5 % m/v), and subjected to an intracardiac injection with a 0.3 ml syringe (31-gauge needle). Between 50 and 100 µL of blood were collected from each individual, placed at -20°C and immediately centrifuged at 10,000 rpm for 3 minutes. Subsequently, red blood cells were separated from serum and stored at -80 C° until downstream analyses.

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol, and DNA concentrations and purity were checked using a spectrophotometer (Nanodrop ND-1000; Thermo Scientific, USA), according to previous recommendations (29). Relative telomere length was measured using real-time quantitative PCR on a BioRad CFX 96 (Bio-Rad Laboratories, Hercules, California, USA), strictly following the protocol recently validated for wild vertebrates by McLennan and colleagues (30).

Statistical analyses

We used the package lme4 in RStudio (Version 1.1.463) to run a linear mixed model including island (Corsica vs. Sardinia) and sampling time (beginning vs. end) and their interaction as fixed factors. Individual identity was included as a random factor to control for non-independence of measurements. The inclusion of sampling location as a random factor did not improve the fitting of the model (i.e., the AIC value was not reduced beyond 2), thus it was no further considered. We used the Tukey test for post-hoc analyses.

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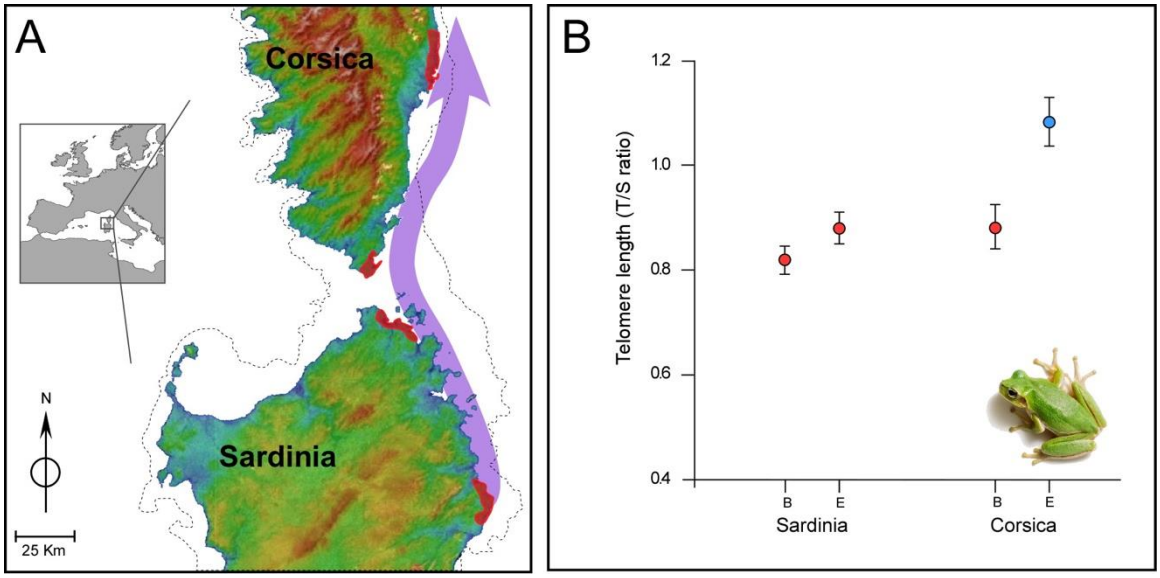
Figures

Figure 1. A) Study area. Red shapes represent the four geographic areas where Tyrrhenian tree frog individuals were collected within Sardinia and Corsica islands. Purple arrow indicates the approximate route of late-Pleistocene range expansion of the Tyrrhenian tree frog, as inferred by previous population genetics, phylogeographic, and species distribution modelling investigations (20, 21). Dashed line shows the coastline during the last glacial maximum (21,000 years ago). B) Values of telomere length in tree frogs sampled twice over a common garden experiment of 12 months. Groups sharing the same colour do not differ significantly from each other. Least square means ± standard errors are shown. B: beginning of the experiment; E: end of the experiment.



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