Università degli Studi della Tuscia di Viterbo

Dipartimento di Scienze Ecologiche e Biologiche

Corso di Dottorato di Ricerca in

Ecologia e Gestione Sostenibile delle Risorse Ambientali - XXXII / XXXIII ciclo

PHENOTYPIC CONSEQUENCES OF A RAMPANT MITOCHONDRIAL INTROGRESSION

(s.s.d. BIO/07)

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A.A. 2019/20

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1. INTRODUCTION

1.1 *The mitochondrial introgression*

Hybridization is a dynamic process that contribute to shape biodiversity, fascinating eco-evolutionary biologists since the very beginning of Darwin's time. Increasing attention is being paid to the dynamics that drive the hybridization process induced by climatic and environmental changes, which have impacted the evolutionary history of populations, species and ecological communities (Chunco, 2014; Brennan *et al.,* 2014; Taylor *et al.,* 2015). One of the most frequent hybridization events among previously isolated populations is the introgressive hybridization, which can lead to mito-nuclear discordances (Toews & Brelsford, 2012; Arntzen *et al.,* 2016; Bonnet *et al.,* 2017; Lin *et al.,* 2018). This discordance occurs when the geographic location and/or extension of contact zones between nuclear genomes do not match with the location and/or extension defined by mitochondrial basis. Massive hybridization events resulting in mito-nuclear discordance occurred after glacial and interglacial periods (mainly Quaternary, Pleistocene), which have been characterized by species range expansions and contractions as a result of adaptation to global environmental changes (Hofreiter & Stewart, 2009; Hewitt, 2011; Feliner, 2011). The numerous examples of mito-nuclear introgression in many taxa (Mallet, 2005; see Toews & Brelsford, 2012 for a review) reveal the eco-evolutionary role of introgressive hybridization in shaping species boundaries and moulding spatial distribution of genetic diversity within species (Harrison & Larson, 2014; Canestrelli *et al.,* 2016a; Pfennig *et al.,* 2016).

Most cases of natural introgression concern the mitochondrial DNA (mtDNA), such as the mitochondrial genotype from a population is incorporated into the gene pool of another divergent population through hybridization and backcrossing of the hybrids with individuals from the parent populations (see Toews & Brelsford, 2012; Canestrelli *et al.,* 2016a and references therein). The reason/s why one genome passes across populations and the other do not is unclear but understanding why in most cases is the mtDNA that introgress can open interesting windows on the eco-evolutionary role of mitochondria. Multiple mechanisms have been proposed to explain this pattern, mainly based on theoretical approaches spanning from random processes – *i.e.,* genetic drift and demographic disparities or hybrid zone movement – to deterministic processes – *i.e.,* sex-biased mating, fecundity and dispersal – (reviewed in Toews & Brelsford, 2012). According to the random process hypothesis, mitochondrial introgression involves neutral markers that flow along the hybrid zone. On the other hand, the deterministic approach is supported by numerous instances of selection acting on mtDNA (Blier *et al.,* 2001; Rand, 2001; Ballard & Dean, 2001; Ballard & Whitlock, 2004; Meiklejohn *et al.,* 2007; Rollins *et al.,* 2016), suggesting that selection may affect the flow of specific single markers on the mitochondrial chromosome or groups of them (via physical and functional linkage), probably due to environment-genome interactions and mito-nuclear co-adaptation/co-evolution (Blier *et al.,* 2001; Rand *et al.,* 2004; Sedghifar *et al.,* 2016). This leads other authors to propose the existence of adaptive introgression via positive selection on mitochondrial genotype (Ballard & Whitlock, 2004; Boratynski *et al.,* 2014; Hedrick, 2013; Bonnet *et al.,* 2017). In this regard, Hill (2019a) suggests that despite the constraints imposed by the mito-nuclear compatibility, the selective introgression of mitochondrial genotype and mito-nuclear genes occurs since the advantageous fitness from introgressing mitochondrial genotypes could compensate mito-nuclear mismatches (Rand *et al.,* 2004; Dowling *et al.,* 2008; Sloan *et al.,* 2017; Hill, 2019a, b). This view underpins the key role of cyto-nuclear epistatic interactions and co-adaptation in shaping

phenotypic variations depending on genome-environment-genome interactions (Paliwal *et al.,* 2014; Zhu *et al.,* 2014; Rand *et al.,* 2018). Insights that came out from this adaptive view of introgressive hybridization suggest that the spread of introgressive hybridization would be regulated by a mix of phenotypic and genetic qualities of hybrids (Canestrelli *et al.,* 2016a, b). Consequently, the identification and the quantitative evaluation of the phenotypic foundation of mitochondrial introgression would be crucial to understand the genotype-phenotype variations that drive mito-nuclaer discordances, contributing to shed more light on the eco-evolutionary processes that generate the current biogeographic and genetic diversity patterns.

1.2 *Dispersal related phenotypic traits*

Dispersal is a key process that shapes spatial patterns of biodiversity at all levels of organization, from individuals to ecosystems (Kokko & López-Sepulcre, 2006; Ronce, 2007; Clobert *et al.,* 2012; Little *et al.,* 2019). In the last decades, eco-evolutionary biologists have been increasingly focused on personality traits that drive dispersal processes, underpinning the role of inter-individual variations of dispersal behaviours in shaping animal movements and space use, thus, determining how species' ranges evolve in their geographic distribution and genetic patterns (Carere & Gherardi, 2013; Canestrelli *et al.,* 2016b). In particular, phenotypic traits such as activity, exploration, boldness, aggressiveness, and sociality have been correlated with greater dispersal (Dingemanse *et al.,* 2003; Cote & Clobert, 2007; Cote *et al.,* 2010a; Debeffe *et al.,* 2013; Hudina *et al.,* 2014; Cote *et al.,* 2017).

Dispersal is one of the most challenging tasks for animals, considering its costs in terms of energy, time, risks and opportunities (Stamps *et al.,* 2005; Ronce, 2007; Bonte *et al.,* 2012; Travis *et al.,* 2012), and results as an outcome of multiple phenotypic traits that often cluster in "dispersal syndromes" together with performance and life history traits (Ronce & Clobert, 2012; Stevens *et al.,* 2014; Cote *et al.,* 2017; but see Bonte & Dahirel, 2016). Despite dispersal syndromes have also been reported to be repeatable, plastic and consistent overtime and across contexts (Gifford *et al.,* 2014; Cosentinoo & Droney, 2016; see Kelleher *et al.,* 2018 for a review), the multicausality of dispersal reveals that it is also strongly condition- and context-dependent (Nathan, 2001; Nathan *et al.,* 2008; Matthysen, 2012; Fronhofer *et al.,* 2018). Indeed it is influenced by the interactions between internal/state factors (phenotypic conditions of the organism *i.e.,* energy storage, morphological structures, hormonal and oxidative status) and external factors (ecological contexts *i.e.,* density and distribution of conspecifics/competitors or predators, food and shelter availability, habitat quality and structure), which can vary during the organisms' lifetime (Massott *et al.,* 2002; Debeffe *et al.,* 2012 Kisdi *et al.,* 2012; Fronhofer *et al.,* 2015). These factors can influence the motivation, the movement patterns and the rate of organisms' dispersal, as well as the trade-offs with other life-history traits according to cost-benefit balances (Ronce *et al.,* 2000; Revilla *et al.,* 2004; Clobert *et al.,* 2009; Cote *et al.,* 2010a, 2013; Baguette *et al.,* 2013; Duputié & Massol, 2013). Such complex systems of interacting factors that shape dispersal processes result in a higher individual variability of dispersal traits that could differ in each phase of the dispersal processes, from the first acquisition of information *in situ* (pre-departure/decision make), the exploration (departure/emigration/wandering) and the long-distance dispersal (transfer/migration/transience), to the arrival (permanent settlement/immigration, Heinz *et al.,* 2006; Dingemanse *et al.,* 2007; Clobert *et al.,* 2009; Stevens *et al.,* 2010; Travis *et al.,* 2012; Arendt, 2015).

1.3 *Locomotor performances*

Animals display a plethora of performances traits to answering to ecologically relevant tasks, most of which include both regulatory activities of physiological processes and dynamic activities of physical movements (*e.g.*, athletic performances). The result is a complex integrative framework of phenotypic traits – morphological, physiological, behavioural – all functionally linked to each other (Careau & Garland, 2012; Lailvaux & Husak, 2014; Irschick & Higham, 2016; Biewener & Patek, 2018). Individuals can show different strategies to perform the same task according to energetic costs/benefits balances (Careau & Garland, 2012; Irschick & Higham, 2016; Husak & Lailvaux, 2017). Accordingly, performances can impose severe trade-offs for the allocation of energetic resources (Salin *et al.,* 2015; Husak *et al.,* 2016; Lailvaux & Husak, 2017 and references therein), and thus contribute to drive the evolution of morphological, physiological, and behavioural traits within energetical and environmental constraints, leading to important eco-evolutionary implications (Irschick & Garland, 2001; Ghalambor *et al.,* 2003; Irschick *et al.,* 2008; Husak *et al.,* 2009).

Among athletic performances, the locomotor performance is one of the most widely studied due to its necessary involvement in many key-survival and reproductive tasks, from foraging/prey capture, predator escape and territory defence, to reproductive success, social interactions and dispersal (Irschick & Losos, 1998; Fitzpatrick *et al.,* 2003; Husak *et al.,* 2006; Irschick & Higham, 2016; Biewener & Patek, 2018 and reference therein). Therefore, locomotor performances are also functionally linked to many life-history traits (*i.e.,* Beck & Congdon, 2000; Ghalambor *et al.,* 2004), resulting to be a good proxy for many physiological and morphological correlates, such as the energetic status/costs, to body size and development (Husak, 2006; Husak *et al.,* 2006; Careau & Garland, 2012; Martin *et al.,* 2015; Denton *et al.,* 2017).

As how organisms use their performance capacities depends on their phenotypic conditions and ecological context (Irschick & Garland, 2001), performances' traits can be subject to many different selection pressures dealing with survival-fitness balances, such as best performers may not always be favoured (Irschick *et al.,* 2007, 2008; Irschick & Higham, 2016 and references therein). In fact, a high individual variation exists in locomotor ability, and this variation can have important eco-evolutionary implications (Elnitsky & Claussen, 2006; Rob *et al.,* 2011; Careau *et al.,* 2014). Consequently, measuring maximal performances is crucial to understand the eco-evolutionary links between maximal locomotory performances, physiology, morphology, and behaviour (Arnold, 1983; Irschick & Higham, 2016; Biewener & Patek, 2018 and reference therein), and can give insight into which selective pressure is acting on performance (Irschick *et al.,* 2008). Maximal locomotor performances are often used as a proxy for evaluate the dispersal capacity of an individual within a population of species (Johnson *et al.,* 2010; Hillman *et al.,* 2014a, b; Rank *et al.,* 2020). To do so, integrating maximal performance measurements, physiological and behavioural data could result in key interpretation on how species use, move and evolve in their habitats, providing insights on micro and macroevolutionary dynamics (Irschick, 2002, 2003).

1.4 *Mitochondrial genotype and dispersal phenotype*

Mito-nuclear genetic variations affect many life-history traits via pleiotropic and epistatic interactions (Rand *et al.,* 2004; Dowling *et al.,* 2007; Ellison & Burton, 2008; Wolff *et al.,* 2014; Hill, 2019b; Havird *et al.,* 2019). Therefore, it has become clear that complex mechanisms of co-variations/co-adaptations between mito-nuclear

genomes shape life-history trade-offs according to metabolic and oxidative constraints (Clancy, 2008; Flatt & Heyland, 2011; Burton & Barreto, 2012; Hill, 2019b; Zhu *et al.,* 2014). However, recently increasing attention has been focused on mitochondrial genotype and its effects on organismal phenotype, investigating the functional and adaptive significance of mitotype and its eco-evolutionary implications (Ballard & Rand, 2005; Gershoni *et al.,* 2009; Ballard & Pichaud, 2014; Breton *et al.,* 2014; James *et al.,* 2016; Wolff *et al.,* 2016). This integrative perspective on mitochondria deeply questioned theories on the neutrality of its genome's variations (Ballard & Kreitman, 1995; Ballard & Whitlock, 2004; Bazin *et al.,* 2006; Dowling *et al.,* 2008; Galtier *et al.,* 2009).

Mitochondria is considered as the "powerhouse and the peacemaker" of the organism due to its key role in managing the metabolic activity, and thus, influencing many life-history trade-offs and fitness key-traits from energy production and allocation, fertility and stress resistance, to behaviour, performance, adaptation, and lifespan (Ballard & Melvin, 2010; Correa *et al.,* 2012; Dobler *et al.,* 2014; Hood *et al.,* 2018; Ghiselli & Milani, 2019; McKenzie *et al.,* 2019). Therefore, it strongly contributes to driving the evolution of life-history strategies within the fast-slow continuum posited by the pace of life syndrome theory (POLS, Dammhahn *et al.,* 2018; Chung *et al.,* 2018; Montiglio *et al.,* 2018). The POLS theory asserts that individuals have a suite of phenotypic traits ranging from metabolism, hormonal and immunity traits, to behaviour, performances and longevity that co-evolve in response to different ecological conditions, defining the life-history strategies, where the link between metabolic rate and personality appears crucial (Réale *et al.,* 2010; Careau *et al.,* 2008; Careau & Garland, 2012). The interconnections between POLS traits and mito-nuclear genotypes mentioned above suggest a functional link between mtDNA, metabolic activity, behavioural and performance traits that might shape the phenotypic and genetic patterns in hybrid zones (Burton *et al.,* 2010; Perkins *et al.,* 2013; Canestrelli *et al.,* 2016a, b). In fact, dispersal has been included within the POLS on the base of energetic constraints (Fjerdingstad *et al.,* 2007; Careau *et al.,* 2009; Stevens *et al.,* 2012, 2013; Le Galliard *et al.,* 2013; Buoro & Carlson, 2014). Accordingly, it was suggested that fast individuals disperse more than slow ones and are characterized by high metabolic rate and activity levels, small sizes but fast growth rate, boldness, risk-taking and explorative attitudes, high aggressiveness and early sexual maturation but short lifespan (Réale *et al.,* 2010; Debeffe *et al.,* 2013; Hudina *et al.,* 2014; Cote *et al.,* 2017). Indeed, high metabolic rates increase the activity level for feeding (Careau *et al.,* 2008; Biro & Stamps, 2010; Killen *et al.,* 2011; Gifford *et al.,* 2014; Myles-Gonzalez *et al.,* 2015), and activity enhances the probability of departure for a novel environment (O'Riain *et al.,* 1996; Belthoff & Dufty, 1998; Pintor *et al.,* 2008). High activity to feed is related with an explorative attitude that enhances the probability of encountering food and/or partners (Debeffe *et al.,* 2013; Gifford *et al.,* 2014; Reader, 2015), while high exploration is related to boldness, which increase the propensity to take risks linked to dispersal (Fraser *et al.,* 2001; Rehage & Sih, 2004; Myles-Gonzalez *et al.,* 2015).

These functional links between physiological, behavioural and performances traits related to dispersal are supported not only by the fact that behaviours and performances are regulated by a common underlying physiological mechanism, such as energetic metabolisms (Careau *et al.,* 2008, 2009; Careau & Garland, 2012), but also by genetic mechanisms (Sih *et al.,* 2004a, b; van Oers *et al.,* 2005b; Penke *et al.,* 2007; Dochtermann & Roff, 2010; Careau *et al.,* 2011; Flatt & Heyland, 2011; Henriksen, *et al.,* 2020). In fact, numerous studies have explored the genetics of dispersal, investigating if dispersal polymorphisms represent different genotypes and providing evidence for the correlations between genes and dispersal traits, the heritability of dispersal traits and selection acting on dispersive genotypes (Dingemanse *et al.,* 2002; Pasinelli *et al.,* 2004; van Oers *et al.,* 2004a; Haag *et al.,* 2005; Sinervo *et al.,* 2006; Gloria-Soria & Azevedo, 2008; Niitepõld *et al.,* 2009; Doligez *et al.,* 2009; Duckworth, 2012; Wheat, 2012;

Zera & Brisson, 2012; Korsten *et al.,* 2013; Cobben *et al.,* 2015; Saastamoinen *et al.,* 2018; Cayuela *et al.,* 2019b). Consequently, dispersal might not involve a random sample of genotypes as was typically assumed, but instead can be enriched for certain genotypes.

Globally these studies have contributed to increasing our understanding of whether dispersal traits co-vary across genetic lineages and showed how genomes can influence dispersal evolutionary rates and outcomes, particularly under non-equilibrium conditions, such as at range expansion edges (Cobben *et al.,* 2015; Canestrelli *et al.,* 2016a, b; Saastamoinen *et al.,* 2018). However, little is known about the variation of phenotypic traits linked to dispersal in relation to mito-nuclear discordance patterns in natural populations. Recent works showed that artificially introgressed mtDNA can affect personality traits (*i.e.,* boldness, exploration, activity) linked to dispersal capacity (Šíchová *et al.,* 2014; Løvlie *et al.,* 2014), suggesting an adaptive mitochondrial introgression.

1.5 *The mitochondrial introgression of the Italian fire salamander*

The fire salamander *Salamandra salamandra* (Linnaeus, 1758) is an ovoviviparous urodele amphibian (Salamandridae, *Salamandra*) widespread in Mediterranean and European temperate forests (Sillero *et al.,* 2014). Phylogeographic studies on the Italian fire salamander *S. salamandra* reveal the presence of two main groups of populations occurring along the Italian peninsula: one endemic to the southern portion of the peninsula, *S. s. giglioli*, and the other, *S. s. salamandra*, with a pre-alpine and alpine distribution (Sillero *et al.,* 2014). These two populations were probably hybridized following a post-glacial range expansion with a secondary contact in late Pleistocene (Steinfartz *et al.,* 2000). However, the nuclear and mitochondrial zones of geographic transition between the two groups are located 600 km from one another, suggesting rampant mitochondrial introgression following the post-glacial secondary contact between lineages (Bisconti *et al.,* 2018).

Mitochondrial introgression has been previously reported in salamanders (Weisrock *et al.,* 2005; Denton *et al.,* 2014; Canestrelli *et al.,* 2014; Johnson *et al.,* 2015). However, no data reported such a huge geographic discordance between mito-nuclear lineages. The insights of a southward expansion of the *S. s. salamandra* to explain such a particular pattern raises the question: "why did the northern *S. s. salamandra* mtDNA introgressed so deeply into the southern *S. s. gigliolii* population?". To answer this question it is necessary to test if the introgressed northern lineage carry adaptive dispersal traits that could have promoted (or be promoted) its rampant introgression.

1.6 *The dispersal of salamanders*

The huge geographic discordance between mito-nuclear lineages showed in the Italian fire salamander is unexpected considering its limited dispersal abilities due to the territorial and philopatric behaviour (Warburg, 1994; Mathis *et al.,* 1995). However, numerous data on salamanders' dispersal from capture-mark-recapture (Rothermel, 2004; Rittenhouse & Semlitsch, 2006; Bar-David *et al.,* 2007; Gamble *et al.,* 2007; Schulte *et al.,* 2007; Schmidt *et al.,* 2014; Lowe, 2010), indirect indices of dispersal (*i.e.,* genetic differentiation, Lowe *et al.,* 2008; Bani *et al.,* 2015; or tracked paths, Pittman & Semlitsch, 2013), and from studies that tested behavioural, physiological or performance traits (Marsh *et al.,* 2004; Shalk & Luhring, 2010; Gifford *et al.,* 2014; Denton *et al.,* 2017; Addis *et al.,* 2019; Lewis & Sullivan, 2020), suggest that they might be more dispersive than expected. At larval stages, downstream dispersal and inter-pond movements of larvae can occur, both actively and passively for resource needs (*i.e.,* feeding

opportunities, refugia), or because of the advective force of the flowing water (Thiesmeier & Schuhmacher, 1990; Lowe, 2003; Cecala *et al.,* 2009; Segev & Blaustein, 2014). However, despite larval phase may represent the very first dispersal stage (Ferguson, 2000), the earlier terrestrial life-stage is supposed to be the highest dispersal phase due to the competition for resources and territory with both adults and contemporary juveniles (Stamps, 1994; Semlitsch, 2008; but see Ousterhout & Liebgold, 2010). Juvenile dispersal is a critical component of amphibian species' ability to colonise new habitat, increase gene flow, and adapt to changing environmental conditions or habitat fragmentation (Rothermel, 2004; Cushman, 2006; Gamble *et al.,* 2007; Griffiths *et al.,* 2010; Osbourn, 2012; Pittman & Semlitsch, 2013; Pittman *et al.,* 2014; Van Drunen *et al.,* 2021). However, comparative studies on dispersal traits across life-stages are missing, and thus little is known of the contribution of each stage to this amphibian dispersal.

1.7 *Aim of the research*

As we have attempted to illustrate here, the integration of personality research methods, eco-physiology, genetic approaches (*i.e.,* genetic architecture and heritability of animal life history syndromes) and bio-logging technologies (*i.e.,* spatial and temporal variation) is allowing to improve our knowledge of the eco-evolutionary dynamics of dispersal processes. In this research project we used a multi-trait approach to study the phenotypic consequences of a rampant mitochondrial introgression. We investigated the genotype-phenotype interactions in relation to biogeographic pattern of mito-nuclear discordance, contributing to the increasing evidence of the functional, adaptive and eco-evolutionary implications of the mito-nuclear genetic variations (*i.e.,* the emerging theory of mito-nuclear ecology, Hill, 2019a and citations therein). In particular, the aim of the research was to experimentally test the hypothesis that the introgressed mtDNA might carry specific, likely adaptive, personality and physiological traits related to dispersal, which may be drivers of mitochondrial introgression in the fire salamander *Salamandra salamandra* within Peninsular Italy. To achieve this aim, we characterised behavioural traits related to dispersal (*i.e.,* activity, boldness and exploration) in the two mitotypes of the fire salamander. Furthermore, we also evaluated developmental traits related to life history strategy (*i.e.,* body size and condition, heart rate after locomotor effort, larval period duration), and we provided a new methodological approach to measure locomotor performance in this amphibian. This is the first experimental study among salamander species exploring different dispersal personality and life history traits between naturally occurring mitotypes along a secondary contact zone.

2. BEHAVIOURAL FOUNDATION OF MASSIVE MITOCHONDRIAL INTROGRESSION IN THE FIRE SALAMANDER, *Salamandra salamandra*

2.1 Introduction

Secondary contact zones are geographic regions where ancient divergent and spatially isolated evolutionary lineages meet after range expansions events, and have long been known as excellent natural systems to investigate eco-evolutionary dynamics between and within species (Hewitt, 1988). When hybridization occurs between the interacting lineages, a wide and dynamic range of spatial patterns of genetic variation can arise, leading to a diversity of hybrid zone structures (Barton & Hewitt, 1985; Abbott *et al.,* 2013). Within this diversity of possible outcomes, considerable attention has been paid to instances of mito-nuclear discordance across contact zones (Toews & Brelsford, 2012; Arntzen *et al.,* 2016; Bonnet *et al.,* 2017; Lin *et al.,* 2018), which consists in geographic location and/or spatial extension of a contact zone between nuclear genomes not matching with that defined by mitochondrial genomes (Toews & Brelsford, 2012).

Multiple mechanisms have been proposed to explain patterns of mito-nuclear discordance. Some authors emphasise the role of random processes, such as genetic drift and demographic disparities or hybrid zone movement (Funk & Omland, 2003; Currat *et al.,* 2008; Krosby & Rohwer, 2009). Other authors emphasise deterministic processes instead, such as sex-biased mating/dispersal or differential fecundity among hybridizing lineages (Petit & Excoffier, 2009; Ng & Glor, 2011; Rheindt & Edwards, 2011). Among the latter, some stressed the role of positive selection and adaptive introgression of mitochondrial genotypes (Ballard & Whitlock, 2004; Boratynski *et al.,* 2014; Hedrick, 2013; Bonnet *et al.,* 2017). Accordingly, events of massive mitochondrial introgression would take place if the receiving lineage acquires fitness advantage from the introgressing mitotype, rather than fitness costs by mito-nuclear incompatibility (Rand *et al.,* 2004; Dowling *et al.,* 2008; Sloan *et al.,* 2017; Hill, 2019a, b). As a matter of fact, mitochondria are the powerhouse and the master switch of the organism (McKenzie *et al.,* 2019) and affect a wide range of life history traits like respiration, metabolic activities, athletic performances, sexual selection, lifespan, and behaviour (Ballard & Melvin, 2010; Breton *et al.,* 2014; Dobler *et al.,* 2014; Hood *et al.,* 2018), which hampers the identification of the exact selective advantage associated to cases of mitochondrial introgression.

Recent studies reported association of mitochondrial genetic variation with personality traits, and suggested a role for adaptive introgression of personality traits associated to mitochondrial DNA (hereafter mtDNA) in explaining differential introgression of alternative mitotypes (Šíchova *et al.,* 2014; Løvlie *et al.,* 2014). Interestingly, personality traits have been shown to contribute substantially to modulate individual dispersal (Dingemanse *et al.,* 2003; Cote & Clobert, 2007; Cote *et al.,* 2010). For example, fast dispersal profiles can be characterized by bold, aggressive, risk-taking, and explorative attitudes (Debeffe *et al.,* 2013; Hudina *et al.,* 2014; Cote *et al.,* 2017). Dispersal is a key process in ecology and evolutionary biology, as it contributes in shaping spatial patterns of biodiversity and their variation over time, at all levels of organization (Kokko & López-Sepulcre, 2006; Ronce, 2007; Clobert *et al.,* 2012; Little *et al.,* 2019). Given the key role of mitochondria in energy production and allocation, a link between interindividual variation at the level of mtDNA and variation in energetically demanding behaviours, such as dispersal, looks especially plausible (Careau *et al.,* 2011; Travis *et al.,* 2012; Le Galliard *et al.,* 2013). As a consequence, introgressed mitotypes could carry adaptive phenotypic features linked to increased dispersal capacity, thus explaining cases of massive mtDNA introgression (Canestrelli *et al.,* 2016a, b). However, while instances of mito-nuclear

discordance have been largely investigated, based on analyses of spatial patterns of genetic variation, we still lack a clear understanding of their phenotypic foundation.

Here, we investigate the phenotypic underpinnings of a striking case of discordance in patterns of geographic variation between mitochondrial and nuclear genes, in the fire salamander *Salamandra salamandra*. Recent phylogeographic and population genetic studies (Bisconti *et al.,* 2018) confirmed previous morphological evidence for the occurrence of two distinct evolutionary lineages along the Italian peninsula, *S. s. salamandra* (the northern lineage) and *S. s. giglioli* (the peninsular lineage; Sillero *et al.,* 2014), and suggested the occurrence of a secondary contact between these lineages. However, while nuclear genetic markers located the contact zone in north-western Italy, phylogeographic data based on mtDNA markers spotted it about 600 km to south, in south-central Italy (Bisconti *et al.,* 2018). This geographic pattern was explained by a massive southward introgression of the northern mtDNA lineage into the nuclear background of *S. s. giglioli*, triggered by the post-glacial formation a secondary contact zone in north-western Italy. Despite mtDNA introgression has been previously reported in salamanders, the geographic extent of this mito-nuclear discordance is way more conspicuous (*i.e.,* Weisrock *et al.,* 2005; Denton *et al.,* 2014; Canestrelli *et al.,* 2014; Johnson *et al.,* 2015). Also, the available data (Bisconti *et al.,* 2018) suggest that the mtDNA contact zone in central Italy occurs within a fully "southern" nuclear background. Thus, the Italian fire salamander provide an excellent study system to investigate the possible phenotypic foundation of massive mtDNA introgression. To this aim, here we concurrently characterized mitochondrial, nuclear and phenotypic behavioural variation within the fire salamander mtDNA contact zone in south-central Italy. Moreover, to investigate putative carry-over effects among distinct life-stages, phenotypic variation was analysed in the same individual across larval and the post-metamorphic life stages, since both these stages - and the associated carry-over effects - can be implicated in the adaptive and non-adaptive processes invoked to account for instances of mito-nuclear discordance.

2.2 Materials and methods

Study species

The fire salamander *S. salamandra* is an ovoviviparous urodele amphibian (Salamandridae) widespread in temperate and boreal forests of Europe (Sillero *et al.,* 2014). Females lay aquatic larvae in small brooks and ponds that after 1-3 months undergo metamorphosis; juveniles become fully terrestrial. Sexual maturation is reached after 2-3 years. Adult salamanders are characterized by black-yellow aposematic colorations (Caspers *et al.,* 2020) and show strong breeding site fidelity and territorial behaviour (Warburg 1994; Mathis *et al.,* 1995). Larvae do not show any circadian activity rhythm, while metamorphosed juveniles and adults are active at twilight and night, with activity patterns associated with humidity levels (Himstedt, 1971; Keen, 1984; Manenti *et al.,* 2017).

Dispersal occurs in both larvae and juveniles: larvae actively and passively move across ponds, due to individual resource needs (*i.e.,* food or refugia, Cecala *et al.,* 2009) or transported by drifting (Thiesmeier & Schuhmacher, 1990; Segev & Blaustein, 2014), while metamorphosed juveniles is the highest dispersal phase, as for most amphibians that become territorial in adulthood (Semlitsch, 2008; but see Ousterhout & Liebgold, 2010).

Sampling and housing

We collected 171 larvae across the secondary contact zone between the two mitochondrial lineages in the Picentini mountains (Bisconti *et al.,* 2018), central Italy (see Supplementary Figure 1 and Table 1). To reduce the probability of kinship, larvae were collected from distinct breeding sites that were few km apart. We monitored the sampling sites since late winter, in order to gain larvae immediately after their deposition. Sampled larvae were immediately transported to the housing facilities in small boxes containing stream water (2 L) and kept in a dark and fresh container. Larvae were then individually housed under controlled condition (temperature 18-20° C, humidity 65-70%, natural photoperiod) at the facilities of Tuscia University. We used plastic baskets (10 x 10 cm) as housing containers, with half terracotta saucer as shelter, all set in collective PVC tanks filled with aerated and dechlorinated soft water (10-12° C). Larvae were fed *ad libitum* 3 days a week with live *Chironimidae* and *Tubifex.* After metamorphosis, juveniles were individually housed in the same room. To house juveniles, we used transparent and micro-perforated plastic boxes (11.5 x 11.5 x 6 cm) as terrariums, containing coconut litter, gravel, dechlorinated soft water and a piece of cork as shelter. Juveniles were fed *ad libitum* with *Acheta domestica* and *Tenebrio molitor*. All the individuals were released in the collection sites after the experiments.

All procedures followed the relevant guidelines and regulations for welfare and were approved by the Italian Ministry of Environment (permit number: 0008275.20-04-2018), the Institute for Environmental Protection and Research (#23501, 23-03-2018) and Regione Campania (#0203190, 27-03-2018). Permission to temporarily house amphibians at the University facilities was granted by the Local Health and Veterinary Centre (A.S.L. Tarquinia, license 050VT427).

To identify the mitochondrial lineage of each individual (*i.e.,* the southern or northern lineage of Bisconti *et al.,* 2018), we collected biological tissue for DNA extraction using skin-swabs (Pichlmüller *et al.,* 2013). Since the potential influence (if any) of the skin-swabbing procedure on behavioural tests cannot be predicted, this procedure was carried out at the end of the experiments. That is, the entire set of experimental tests was blind to the mtDNA identity of the studied individuals. Before swabbing, individuals were anaesthetized by submersion in a 0.015 m/v solution of Tricaine methanesulfonate (MS222). Then, swabs were stored in ethanol 96 ° and stored at -20°C until

DNA extraction.

Genotyping

DNA extractions were performed using the standard cetyltrimethylammonium bromide protocol (Sambrook *et al.,* 1989). mtDNA haplotypes were assessed using the polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP) diagnostic technique. Diagnostic RFLP profiling was selected by digesting in-silico all the mitochondrial haplotypes from the northern and southern lineages retrieved in Bisconti *et al.,* (2018) using REPK (Collins & Rocap, 2007), defining two sub-groups (northern and southern haplotypes) and maintaining the default options for defining diagnostic restriction profiles. REPK identified the enzyme *HaeIII* as determining a diagnostic profile in the Cytochrome B (CytB) gene fragment. Then, for all the individuals a 722 bp portion of CytB was amplified by PCR, following the protocol of Bisconti *et al.,* (2018), and subsequently digested by *HaeIII* (Promega) according to the manufacture instructions. Restriction fragments were then separated on 1.5% agarose gel and visualized using UV illumination. Ten specimens from Bisconti *et al.,* (2018) representative of the two lineages were used as reference controls.

To assess the effect of genetic structure of populations on correlative analyses, all the individuals were genotyped at thirteen microsatellites loci (*SST-A6-I, SST-A6-II, SalE-14, SST-B11, SST-G9, SalE12, SalE2, SST-C3, SalE5, SalE7, Sal29, Sal3, SalE06, SalE8*), following the protocols described by Steinfartz *et al.,* (2004) and Hendrix *et al.,* (2010). Forward primers were fluorescently labelled and PCR products were electrophoresed by Macrogen Inc. on an ABI 3730xl genetic analyser (Applied Biosystems) with a 500-HD size standard. Microsatellite raw data were analysed using GeneMapper® 4.1. Individuals with more than 50% of missing data were excluded from the subsequent analyses. Micro-Checker 2.2.3 (Van Oosterhout *et al.,* 2004) was used to test for the presence of null alleles and large-allele dropout in our data. Allelic frequencies were then computed with GENETIX 4.05 (Belkhir *et al.,* 2004) and FSTAT (Goudet, 1995) was used to test for deviations from the expected Hardy-Weinberg and linkage equilibria, using the Bonferroni correction for multiple tests.

The extent of patterns of populations genetic structure within the study area was evaluated by means of the Bayesian clustering method implemented in STRUCTURE (Pritchard *et al.,* 2000). The analysis was carried out using the admixture model, without using sampling location as prior information. Preliminary analyses were conducted to assess model performance, with 80000 steps (the first 20000 discarded as burn-in) and 3 replicates for each K value (*i.e.,* the number of clusters) between 1 and 4. The final analysis contained 10 replicates for each K value, with each run of length 200000 steps, discharging the first 50000 steps as burn-in. The optimal value of K was selected by means of the ln Pr(X|K) method (Pritchard *et al.,* 2000) and the Evanno ΔK method (Evanno *et al.,* 2005)

Behavioural assays

Personality traits linked to dispersal were assessed on either larvae and juveniles: activity, boldness and exploration (see Supplementary Table 2 for a summary). Larvae were tested after one week of acclimatization upon arrival and juveniles were tested three months after the metamorphosis. Each behavioural assay was performed twice, one week apart, to check for repeatability and minimize the effect of potential developmental changes (Bell *et al.,* 2009). All individuals were fasted 48 h before the tests. During the tests room temperature was kept at 20° C and room humidity at 70%. Each behavioural test was recorded using digital cameras (see next paragraphs for details) and videos were analysed manually with the software BORIS (Friard & Gamba, 2016).

(i) Spontaneous activity of larvae. The spontaneous activity in the familiar environment (hereafter FE) was measured in the housing container by scan sampling (Réale *et al.,* 2007). We recorded the activity events with GoPro cameras at predetermined time intervals (2 min/hour, from 10:00 am to 18:00 pm, total 18 min). We also recorded the sheltering behaviour in terms of number of times the animal was inside the shelter with the four paws in.

(ii) Boldness and exploratory activity of larvae. The boldness and the exploration were measured in the unfamiliar novel environment (hereafter NE). Boldness was quantified by measuring the latency to exit from the shelter, and exploration was quantified in terms of time spent in locomotor activity on the total test duration (exploratory activity % on 10 min). NE is assumed to represent a risky situation (Réale *et al.,* 2007) thus, high activity levels and short latencies are associated with an exploratory and bold personality (Beckmann & Biro, 2013; Perals *et al.,* 2017). We also recorded the sheltering behaviour as in the FE. An individual was considered in immobility when had not changed position for more than 3 seconds. The test arena consisted in glass tank (24 x 19 cm) filled with dechlorinated soft water (2 L, 12° C) and containing a plastic leaf with a stone on the top and a plastic duplicate of salamander larvae, all set at opposite corners between each other. Each larva was gently inserted in a PVC tube as shelter and kept for 5 min at dark before open the tube. We tested four individuals simultaneously, avoiding visual contact among them by covering the external walls of the tanks. To remove outliers, individuals that have never exit from the shelter or that have immediately exit from shelter (latency < 1 s) were removed from subsequent analysis.

(iii) Spontaneous activity of juveniles. The spontaneous activity in the FE was measured in the housing container by scan sampling. We took a picture of the arena each 15 min for 24 consecutive hours (total 96 observations), recording any change of position between each consecutive frame (occurrence of activity events). We also reordered the sheltering behaviour as defined for larvae. To ensure a clear view of the animals, the coconut litter was substituted with a white wet paper towel. Animals were kept in this condition for one week before the test. We used four infrared security cameras to record videos, and testing four individuals simultaneously per camera (total 16 individuals simultaneously). The external walls of the boxes were covered to avoid visual contact among individuals tested simultaneously.

(iv) Exploratory activity of juveniles. The exploration was measured in the NE. Exploration was quantified in terms of percentage of time spent in locomotor activity on the total test duration (exploratory activity % on 1 h). An individual was considered in immobility when had not changed position for more than 3 seconds. We also recorded the sheltering behaviour as defined for larvae. The arena set was the same of the FE (see above) and the novelty situation was considered as a sudden change in conditions represented by renewing each paper towel and changing the water container and the shelter before starting the trial. We used four infrared security cameras to record videos testing four individuals simultaneously per camera, avoiding visual contact with other individuals by covering the external walls of the boxes.

Statistical analysis

We calculated the individual based repeatability (intraclass correlation coefficient, therein R_{ICC}) across the two trials of the same test using a two-way mixed alpha model for consistency at single measures (confidence interval 95%). Activity % in the NE of both larvae and juveniles was square root transformed to standardize the data.

To test the effect of mitotype (main factor) we ran a series of linear mixed models (LMMs; factorial, sums of squares, type III, confidence interval 95%). Each variable that was significantly repeatable was entered singly for each

life-stage, using the value of the first trial. The models were fitted with the maximum likelihood (ML) method. To test for consistency of the mitotype effect across life-stages (larval and metamorphosed) on behavioural traits, we ran the same LMMs for repeated measures including subjects as random effect and life-stage as additional within subject factor. Finally, we evaluated the relationships between traits both within and between life-stages using the Spearman rank correlation test of repeatable behavioural traits (confidence interval 95%). All the analysis were performed using SPSS v.23. The graphs were drawn using the software Canvas 11 (ACD Systems of America, Inc.)

2.3 Results

Genotyping

According to the PCR-RFLP diagnostic profile produced by the restriction enzyme *HaeIII* on the CytB gene fragments, 83 out 171 individuals showed northern lineage mitotype, and 88 showed the southern lineage mitotype.

The microsatellite dataset comprised multilocus genotypes for 171 individuals at thirteen loci. Micro-Checker 2.2.3 detected no traces of null alleles and large-allele dropout in our data. There were no significant deviations from Hardy-Weinberg or linkage equilibria after applying the Bonferroni correction for multiple tests. The number of alleles per locus ranged from 2 (*SST-G9*) to 13 (*SalE2*). The analyses conducted in STRUCTURE clearly showed that K=1 best fit the data. Indeed, although both the ln Pr(X|K) and the Evanno's methods addressed 3 as the best clustering option, the inspection of the bar plots for all putative K values revealed no traces of biologically meaningful population structures for K>1. Results from the Bayesian clustering analysis performed by STRUCTURE on the 13 loci are showed in Supplementary Figure 2.

Behavioural assays

The behavioural traits analysed were significantly repeatable at the individual level across test trials for the mobility events in the spontaneous activity in the FE for both larvae an juveniles (R_{ICC} 0.379 and 0.497, respectively, p values 0.000), for the sheltering behaviour of juveniles in FE (R_{ICC} 0.207, p 0.01), for the activity level and the latency to exit from the shelter in NE for the larvae (R_{ICC} 0.214 and 0.310, p 0.030 and 0.003, respectively), and for the activity level and the sheltering behaviour of juveniles (R_{ICC} 0.271 and 0.259, p 0.010 and 0.037, respectively). On the contrary, the measures of behavioural traits across test trials resulted not repeatable for the sheltering behaviour of larvae in both FE and NE (> 0.1).

Results from the behavioural assays showed consistent differences in behavioural traits among the two alterative mitotype. In fact, the effect of mitotype was significant for all the tests in both the life-stages, except for the spontaneous activity level of larvae in the NE, the activity level and the sheltering behaviour of juveniles in the NE. Individuals of the northern mitotype took longer time to exit from the shelter and showed lower levels of exploratory activity in the NE than individuals of the southern one. Individuals of the northern mitotype had lower spontaneous activity level and used more the shelter in the FE then the southern one. The results of the analysis on behavioural traits for larvae and juveniles are summarized in Table 1. Means values of each trait are showed in Figure 1 and 2.

Consistency of mitotype effect across life-stages

Results of the analysis across life-stages are reported in Table 2. The mitotype effect was still present across life-stages in both spontaneous and exploratory activity level both in the FE and in the NE. Individuals of the northern mitotype showed lower spontaneous and exploratory activity levels both as larvae and as juveniles, and in both the NE and the FE. On the contrary, the southern mitotype showed higher spontaneous and exploratory activity levels in both the life-stages and in both the NE and the FE. Overall, both spontaneous and exploratory activity levels were higher in juvenile than in larval stage, but no significant effect of the interaction between mitotype and life stages was found for the activity level in both the FE and NE.

Relationships between traits

We found no significant correlations among behavioural traits in larvae. However, we found negative correlation between the spontaneous activity of juveniles in the FE and the sheltering behaviour of juveniles in both FE (rS = – 0.236; p = 0.007) and NE (rS – 0.211; p = 0.016). We also found the spontaneous activity of juveniles in the FE positively correlated with the exploratory activity in the NE (*i.e.,* less active juveniles used the shelter more than highly active ones, remaining less active in both FE and NE context and *vice versa*; rS 0.211; p = 0.016). Finally, the latency to exit of larvae in the NE was positively correlated with the sheltering behaviour of juveniles in the NE (rS = 0.295; p = 0.006).

2.4 Discussion

We provide experimental evidence that two alternative mitotypes of fire salamander spanning the secondary contact zone in central Italy are associated to two distinct suites of behavioural traits, and that such distinctiveness are consistent across life-stages. In fact, northern mitotype showed a slow-thorough profile as in larvae than in juveniles, while the southern mitotype showed a fast-superficial profile. Still, such a strong phenotypic differentiation is explained by mitotype in the face of no substantial genetic differentiation at nuclear genome, as showed by the analyses at multiple microsatellite loci, supporting the hypothesis that one mitotype could carry adaptive phenotypic traits linked to dispersal driving its rampant introgression (Bisconti *et al.,* 2018).

Fast dispersal profiles have been widely reported at the range-edge of expanding populations, suggesting a combination of spatial sorting and natural selection of specific traits that allow successful invasions (Phillips *et al.,* 2010; Shine *et al.,* 2011; Chuang & Peterson, 2016). For example, the invading populations of cane toads *Rhinella marina* (Gruber *et al.,* 2017) and the house sparrow *Passer domesticus* (Liebl & Martin, 2012) are more exploratory and bolder than those living in long established areas, while expanding populations of the damselfly *Coenagrion scitulum* resulted more active (Therry *et al.,* 2014). On the contrary, we found a slow-shy profile associated with the introgressed northern mitotype, both in larvae and juveniles. The longer latency to exit from the shelter and the lower exploratory activity in the novel environment shown by the northern larvae outline shy personality profile, expressed by long decision-making times in facing the risks of an unfamiliar situation, resulting in a cautious exploration and risk-taking attitude. A similar pattern was expressed by the northern mitotype after metamorphosis, with higher use of the shelter and lower activity in the familiar environment. This in turn outlines a prudent dispersal strategy, which might reflect a reactive coping style. According to the copying style theory, reactive individuals are "slow-thorough" explorers (*sensu* Koolhaas *et al.,* 1999) as they tend to rely more on the information on current environmental conditions, which may take time to acquire, resulting in a cautious, but accurate strategy of exploration (Koolhaas *et al.,* 2010; Groothuis & Carere, 2005; Réale *et al.,* 2010). This could favour the establishment on good feeding grounds, due to a more efficient energy balance, and the capacity of performing prolonged efforts (Verbeek *et al.,* 1994; Deerenberg *et al.,* 1998; Coppens *et al.,* 2010), resulting in higher fitness in stressful situations such as the dispersive phase in novel environments (Cockrem, 2007; Coppens *et al.,* 2010).

The substantial consistency between juveniles and larvae behavioural profiles across context within life-stages and across life stages, as shown by the correlation between the latency to exit from the shelter of larvae and the sheltering behaviour of juveniles, outlines a "structural consistency" for a slow-shy syndrome independent of the life-stage. Repeatability and consistency in behaviour profiles over time and across contexts have been reported in many taxa, including salamanders (Gifford *et al.,* 2014; Cosentinoo & Droney, 2016; see Kelleher *et al.,* 2018 for a review), but less is known about the extent of consistency across distinct life stages (Koenig & Ousterhout 2018; see Cabrera *et al.,* 2021 for a review). Consistency of syndromes that could functionally link physiology and behaviours across contexts and ontogenetic changes suggests common underlying mechanisms, such as metabolism, which could affect energy allocation to growth in both larval and juvenile stages (Biro & Stamps, 2010; Gifford *et al.,* 2014). This pattern suggests that these personality traits could cluster together with some other life history traits, such as metabolic activity, body size and life span in "dispersal syndromes", as observed in many other taxa (Ronce & Clobert, 2012; Stevens *et al.,* 2014; Cote *et al.,* 2017). Indeed, fast dispersal profiles are usually characterized by boldness, aggressiveness, risk-taking and explorative attitudes coupled with small size, fast growth rate, high metabolic rate and

activity level, early sexual maturation and short lifespan (Réale *et al.,* 2010; Hall *et al.,* 2015; Rödel *et al.,* 2015; Dammhahn *et al.,* 2018). In the case of *S. salamandra*, the behavioural profile could remain stable across metamorphosis because larvae are expected to allocate energy to grow and reach optimal size to metamorphose and juveniles to reach sexual maturity. Furthermore, different life-history trade-offs affecting development and survival during early life-stages may also affect traits expressed later in adulthood (carry-over and latent effects, John-Alder & Morin, 1990; Chelgren *et al.,* 2006; Brodin, 2009). Consequently, changes in one behavioural trait could induce contemporary or later maladaptive changes in another. Thus, stable relationships between traits over the entire lifetime could be beneficial to optimize the individual's growth, reproductive success and survival (correlational selection hypothesis, Sih *et al.,* 2004b; Bell, 2007; Han & Brooks, 2013). However, more data from morphological, physiological and performance traits are needed to corroborate this hypothesis.

Our data are in agreement with other studies in several species reporting slow-thorough and long-distance dispersal phenotypes with less active and exploratory attitude, shy, fearful and prudent personality (*i.e.,* Myers & Krebs, 1971; Armitage, 1986; Selonen & Hanski, 2006; Von Merten & Siemers, 2012). Individuals of the northern mitotype, with a prudent personality and a slow syndrome at early life stages, could have higher survival while dispersing in new patches, minimizing the risks and compensating the uncertainties of reproductive success through high adult survival (Stamps, 2007; Lowe, 2010; Vetter *et al.,* 2016; but see Smith & Blumstein, 2008). Consequently, such alternative dispersal strategy might be not so unusual as expected, as slow-thorough explorers might perform better in unstable (unpredictable) environments typically encountered during dispersal (Myers & Krebs, 1971; Clobert, 2009; Careau *et al.,* 2009; Guillette *et al.,* 2011).

The "slow-thorough" dispersal syndrome as potential driver of mitochondrial introgression

Our results support the hypothesis that the mitotypes could carry specific, potentially adaptive life history traits linked to the individual dispersal attitude that allow their permeation irrespective of the nuclear genome across a secondary contact zone. In the case of the Italian fire salamander, the "slow-thorough" northern mitotype carries specific, potentially adaptive dispersal traits that could have favoured the introgression of its mtDNA into the southern lineage *S. s. giglioli*. The charge to the mitochondrial genotype for the differences in dispersal syndrome's traits between the two populations of fire salamanders is supported also by lack of any insights for genetic structure at microsatellite nuclear loci in the study area. Thus, the slow-thorough mitotypes could be the possible driver of rampant mitochondrial introgression in the fire salamander of peninsular Italy. Further genetic investigations at the scale of the whole genome should be addressed to investigate the functional genes linked to the molecular pathways drove by the mitochondrial genes, and the geographic structure of their variation across the Italian fire salamander secondary contact zone.

The key role of behavioural polymorphisms in explaining biogeographic patterns is being increasingly recognized, with the hypothesis that divergent evolution of personalities due to different selection pressure along the range expansion could lead to a non-random distribution among populations of multiple dispersal strategies and associated genotypes (see Canestrelli *et al.,* 2016a, b and citations therein). However, from the first idea that individuals characterized by fast behavioural profiles are the main contributors for a successful range expansion, a more complex scenario is emerging, which can explain the intriguing patterns of differential introgression after hybridization between distinct evolutionary lineages, such as mito-nuclear discordances across secondary contact zones. In fact, distinct strategies expressed along the continuums of fast-slow, superficial-thorough, proactive-reactive

sets of phenotypic traits within dispersal syndromes, can contribute to both establishment and reinforcement of new populations in new areas (Bowler & Benton, 2005; Duckworth, 2008; Clobert *et al.,* 2009; Cote *et al.,* 2017). The coexistence of those dualisms and their intermediate modulations ensure at the same time the first spatial exploration and colonization wave, charged by fast individuals at the expansion front of invading population and the subsequent return to the equilibrium of dispersal rate and population dimension values by their hybridization with the slow individuals of the resident population or by the hybridization between the slow-through rears of the invading population with the resident, allowing the permeation of slow genotypes in both cases (Cobben *et al.,* 2015; Canestrelli *et al.,* 2016b).

2.5 Tables and figures

Table 1. Outcomes of the LMMs for behavioural traits overtime in larvae and juveniles. F = fixed effects estimates. p values (p) associated with the confidence interval of 95% are reported. Significant values concerning genotype effect ($p < 0.005$) are highlighted in bold. Familiar environment (FE), novel environment (NE).

	Life-stage					
	Larvae			**Juveniles**		
Variable	**Effect**	**F**	**p**	**Effect**	**F**	**p**
Spontaneous activity in FE						
Mobility events	mitotype	2.310	0.132	mitotype	10.584	**0.001**
Sheltering				mitotype	27.277	**0.000**
Exploration and boldness in NE						
Latency to exit (s)	mitotype	3.925	**0.050**			
Activity % (sqrt)	mitotype	6.615	**0.011**	mitotype	1.938	0.166
Sheltering				mitotype	2.336	0.129

Table 2. Outcomes of the LMMs for behavioural traits across life stages. F = fixed effects estimates. p values (p) associated with the confidence interval of 95% are reported. Significant values concerning genotype effect ($p < 0.005$) are highlighted in bold. Familiar environment (FE), novel environment (NE).

Variable	**Effect**	**F**	**p**
Spontaneous activity in FE			
Mobility events	mitotype	10.988	**0.001**
	life-stage	95.105	0.000
	mitotype x life-stage	0.039	0.844
Exploration and boldness in NE			
Activity % (sqrt)	mitotype	7.394	**0.007**
	life-stage	34.464	0.000
	mitotype x life-stage	0.379	0.539

Figure 1. Behavioural traits of fire salamander larvae in familiar (FE) and novel environment (NE). (a) Spontaneous activity of larvae in FE expressed as activity events in 18 min; (b) Latencies to exit of larvae in NE; (c) Exploratory activity of larvae in the NE expressed as activity % in 10 min (sqrt). Values are means per mitotype ± standard error (SE). See Supplementary Table 2 for a detailed description of measured variables.

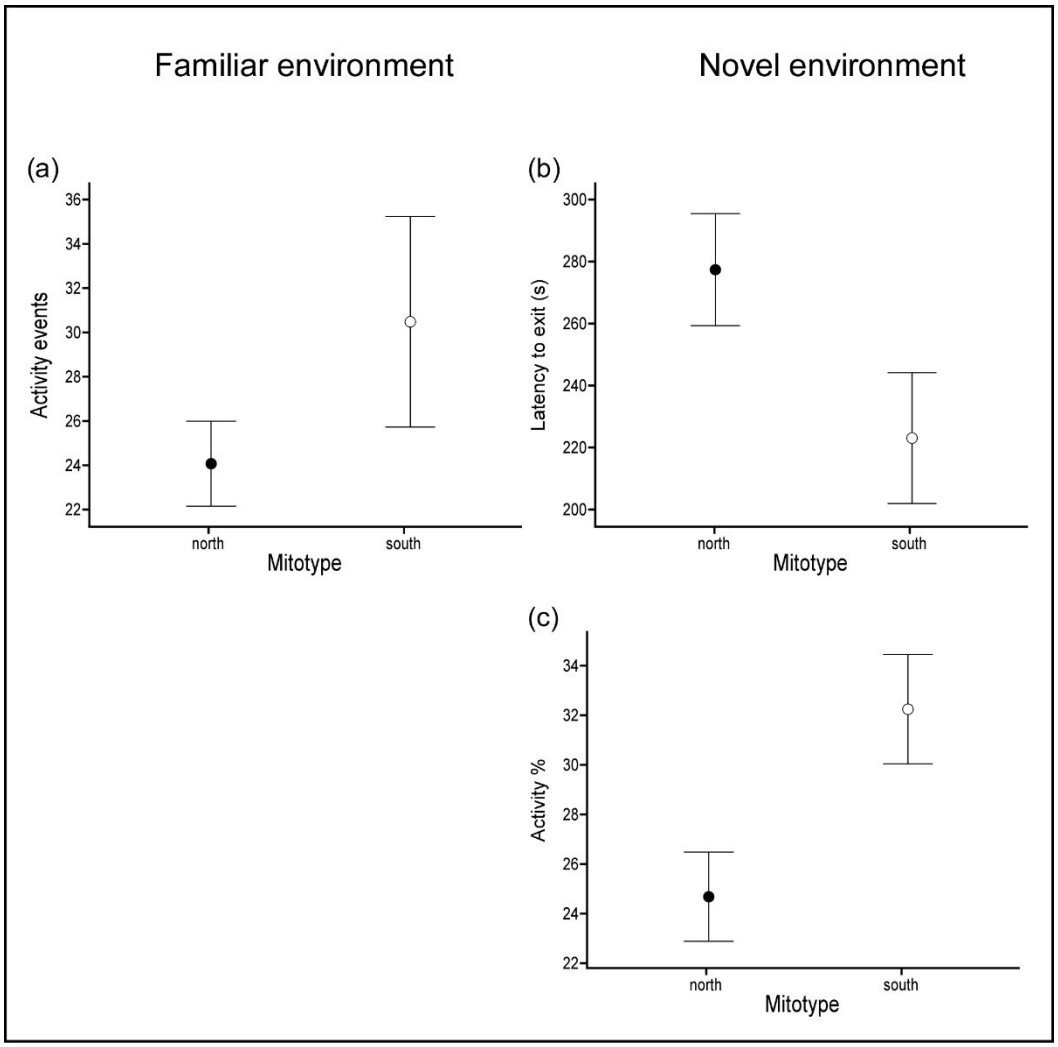
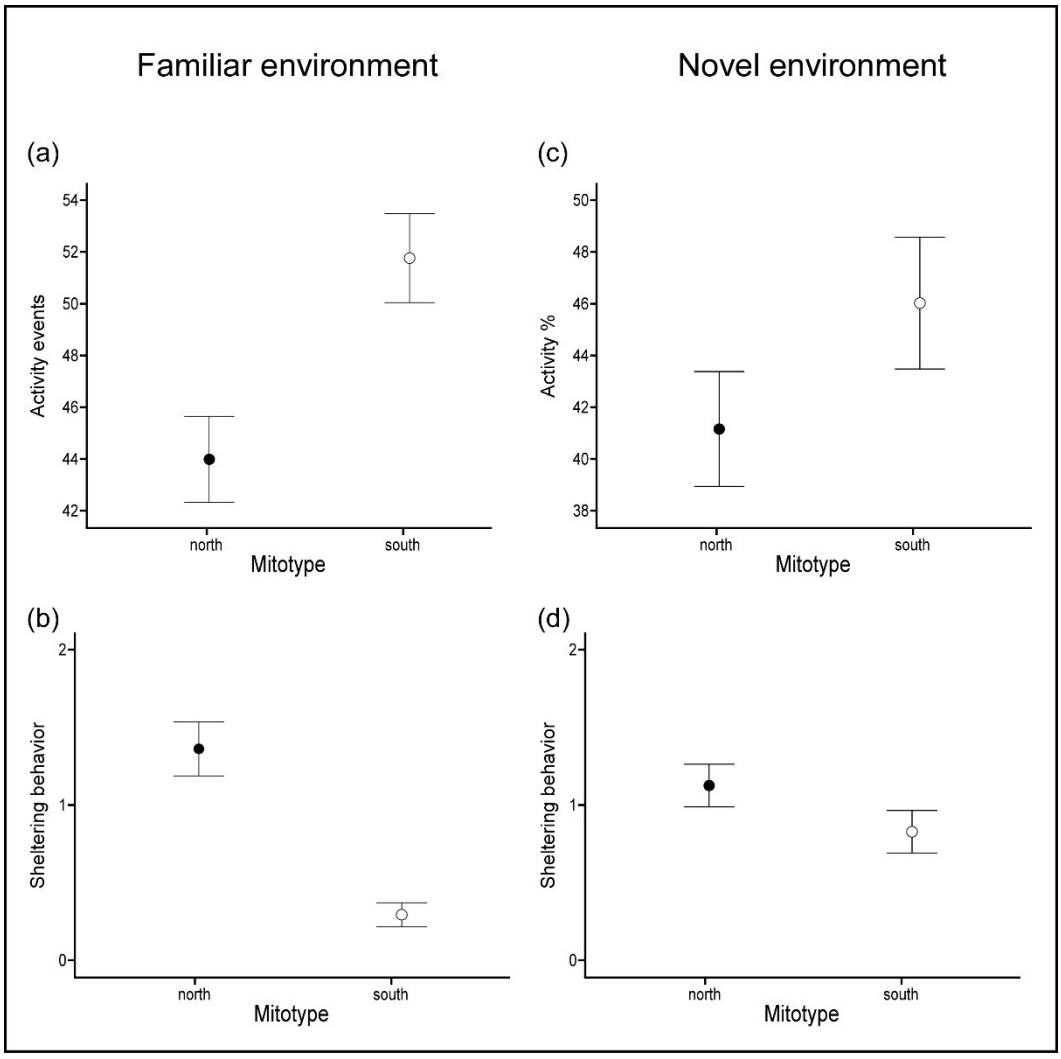


Figure 2. Behavioural traits of fire salamander juveniles in familiar (FE) and novel environment (NE). (a) Spontaneous activity of juveniles in FE expressed as activity events in 24 h; (b) Sheltering behaviour of juveniles in the FE expressed as sheltering behaviour in 24 h; (c) Exploratory activity of juveniles in the NE expressed as activity % in 1 h (sqrt); (d) Sheltering behaviour of juveniles in the NE. Values are means per mitotype ± standard error (SE). See Supplementary Table 2 for a detailed description of measured variables.



2.6 Supplementary materials

Figure 1. A) The fire salamander *Salamandra salamandra*. B) Geographic location of the sampling area where fire salamanders' larvae where collected (selected area in the zoomed map).

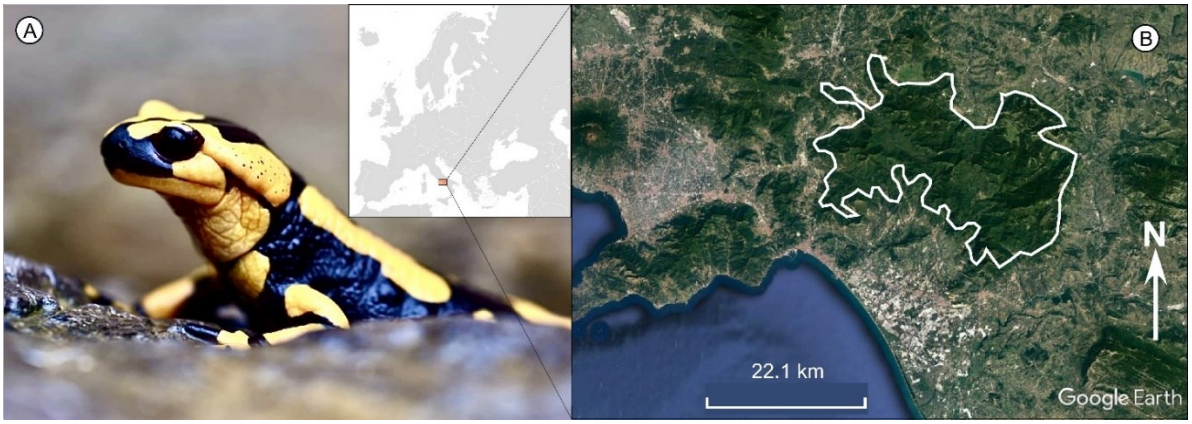


Figure 2. Results from the Bayesian clustering analysis performed by STRUCTURE on the 13 microsatellite loci (details in the main text; the bar plots show the admixture proportions of each individual for the genetic clusters (K) ranging from 1 to 5.

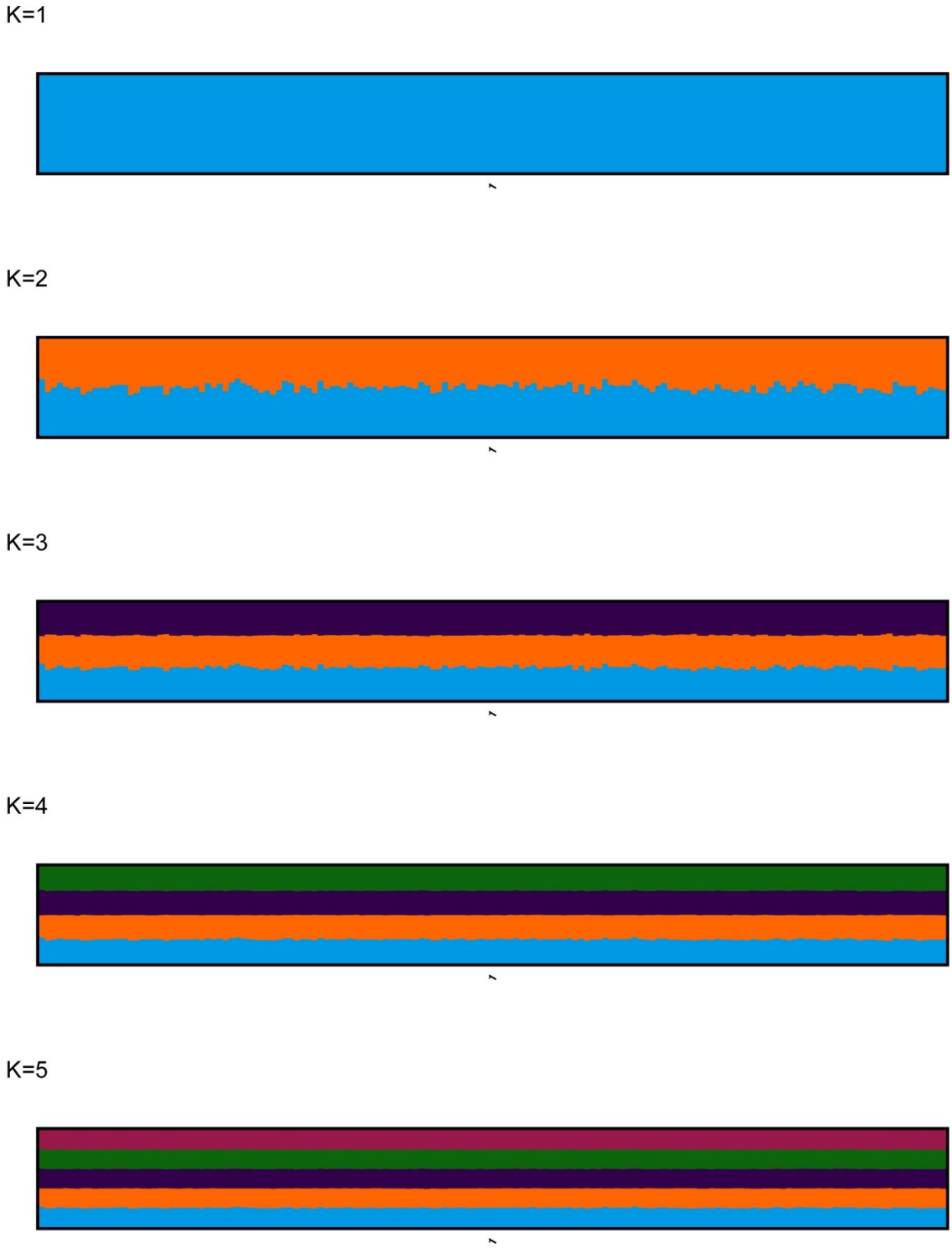


Table 1. Geographic coordinates of the sampling sites (sexagesimal degrees, DMS).

Name	Latitude	Longitude
Montella	40°45'32.64"N	15°2'16.38"E
Giffoni	40°46'4.32"N	14°59'34.14"E
	40°44'33.00" N	14°59'32.00" E
Piani di Verteglia	40°49'15.72"N	14°58'32.16"E
	40°48'52.56"N	14°58'36.48"E
Serino	40°48'5.76"N	14°53'15.18"E

Table 2. Summary of the tests used to evaluate personality traits in larvae and metamorphosed juveniles of the two mitochondrial lineages. Familiar environment (FE), novel environment (NE).

Test	Life-stage	Duration	Variables	Description
Spontaneous activity in FE	Larvae	2 min/h for 9 hrs for a total of 18 min	Mobility events	Number of times the animal makes any locomotor activity (walking and/or swimming and/or change of position)
			Sheltering	Number of times the animal is inside the shelter with the four paws in
	Juveniles	One observation each 15 min, for 24 hrs, for a total of 96 observations	Mobility events	Number of times the animal makes any locomotor activity (walking and/or swimming and/or change of position)
			Sheltering	Number of times the animal is inside the shelter with the four paws in
Boldness and exploratory activity in NE	Larvae	10 min	Latency to exit	Time (s) to exit of the shelter with the four paws out
			Activity %	Percentage of time spent in locomotor activity (walking and/or swimming and/or change of position)
			Sheltering	Number of times the animal is inside the shelter with the four paws in
	Juveniles	1 h	Activity %	Percentage of time spent in locomotor activity (walking and/or change of position)
			Sheltering	Number of times the animal is inside the shelter with the four paws in

3. MEASURING ATHLETC PERFORMANCES IN POST-METAMORPHIC FIRE SALAMANDERS

3.1 Introduction

Athletic performances are key abilities in animals' life since they predict individual success in ecological contexts (Biewener & Patek, 2018; Irschick & Higham, 2016). They consist of dynamic movements that are physically challenging, allowing animals to interact with other conspecific and heterospecific organisms, and with the physical environment (Biewener & Patek, 2018; Irschick & Higham, 2016). These abilities stem from a complex integration of multiple phenotypic traits – *e.g.,* morphological, physiological, and behavioural -, that dictate animal survival and fitness in a wide range of contexts (Careau & Garland, 2012; Lailvaux & Husak, 2014; Irschick & Higham, 2016). Consequently, the comparative study of maximal individual performances in athletic tasks can offer an excellent window on the diversity and evolution of functional phenotypic traits within and among species, and on the role of athletic abilities in moulding the evolutionary pathways of intra- and interspecific lineages in response to significant changes of their physical and biotic environment (Finkler & Claussen, 1999; Husak & Fox, 2006; Irschick *et al.,* 2008; O'Donnell & Deban, 2020).

Among athletic abilities, locomotor performance has been extensively studied, due to its direct implication in a particularly wide range of ecological tasks, such as predator escape, prey capture, territory defence, reproductive success, dispersal, and so forth (Irschick & Losos, 1998; Fitzpatrick *et al.,* 2003; Husak *et al.,* 2006; Irschick & Higham, 2016; Biewener & Patek, 2018 and reference therein). Traditionally, the bulk of studies investigating the evolution of locomotor traits have been focused on analysing the origin (*e.g.,* convergent vs independent) and the evolutionary trends in animal locomotion along the tree-of-life (Biewener & Patek, 2018 and reference therein). However, more recently, increasing attention is being focused on studying inter-individual differences in locomotor performances within and among populations within species (Elnitsky & Claussen, 2006; Rob *et al.,* 2011; Careau *et al.,* 2014). In fact, there is an increasing awareness that athletic abilities can contribute to shaping the eco-evolutionary processes implicated in the genesis of biogeographic patterns both above and below the species level (Irschick *et al.,* 1997; Kosmala *et al.,* 2017; Llewelyn *et al.,* 2010). Among vertebrates, much research effort has been devoted to the study of athletic abilities in the lizards of the genus *Anolis* (Irschick, 2000; Toro *et al.,* 2004; Vanhooydonck *et al.,* 2006) and cane toad *Rhinella marina* (Phillips *et al.,* 2006; Cabrera–Guzmán *et al.,* 2013; Hudson *et al.,* 2016a; Hudson *et al.,* 2020). The integrative analysis of jumping performances in these study systems (based on biomechanical, physiological, and morphometric traits; Irschick *et al.,* 2005a; Hudson *et al.,* 2016a) highlighted an unexpected amount of inter-individual diversity in performance abilities, and suggested a major role of these abilities in shaping the evolution both of dispersal capacities and the species range, over multiple spatial and temporal scales (Phillips *et al.,* 2010; Hudson *et al.,* 2016a; Hudson *et al.,* 2020; Vanhooydonck *et al.,* 2006). However, despite the increasing realisation of the potential insights coming from the study of inter-individual differences in animal performance traits, this study is in many cases hampered by species-specific lifestyles, such as in species that are elusive and tend not to react impulsively to external stimuli. In these species, identifying and directly quantifying genuinely informative athletic performance traits could be challenging.

The European fire salamander *Salamandra salamandra* (Figure 1 A) is a urodele amphibian widely distributed across much of the Western Palaearctic, where it inhabits mixed, moist deciduous forests from the sea level up to 2500 m (Lanza *et al.,* 2009; Sparreboom, 2014). It is a nocturnal and crepuscular dweller, mostly active on rainy nights,

whereas it spends day-time under moisty woods, stones or the leaflitter (Lanza *et al.,* 2009). Once the metamorphosis of the aquatic larvae has been completed, the fire salamander becomes strictly terrestrial, with only females approaching water to lay larvae. In the adult phase, this species shows a marked elusive lifestyle. When found in the aboveground its movements are slow and, even when stimulated by a (natural or mimicked) predator, its defensive behaviour is rather static, characterized by remaining motionless, usually without attempting to escape predator attacks (it possesses antipredator toxin secretions; Brodie *et al.,* 1974; Brodie, 1977; Lüddecke *et al.,* 2018; Preißler *et al.,* 2019).

Here we present a simple, rapid and low-cost method to quantify athletic performance abilities in post-metamorphic fire salamanders accurately. We aim to provide a test that could force this elusive, and apparently unreactive species, to perform at its extreme limit, allowing the analysis of athletic performance to become part of the extensive body of literature concerning patterns of phenotypic diversity, distribution, and evolution in this species (Steinfartz *et al.,* 2000; Bisconti *et al.,* 2018).

3.2 Methods

Sampling and housing

We collected 78 fire salamander individuals at the larval stage immediately after deposition to standardise the entire experimental procedure. At this aim, we monitored breeding sites in the Picentini Mts. (Campania Region, Italy) between March and June in 2019, when the larvae laying usually occurs (Sparreboom, 2014). Collected larvae were individually housed under controlled and standard conditions in a humid chamber (room temperature 18-20° C, humidity 65-70%, natural photoperiod) at the facilities of the Department of Ecological and Biological Sciences of the University of Tuscia (Viterbo, Lazio, Italy). Salamander larvae were individually housed within pierced plastic baskets (10 x 10 x 10 cm), each with a terracotta saucer used as shelter. All plastic baskets were placed collectively within a PVC tank filled with aerated soft water (dechlorinated and osmotic waters 1:1) and water temperature monitored using a temperature data logger (Hobo Pendant MX2201). Larvae were fed *ad libitum* three days a week with live tubifex and chironomid larvae. Once metamorphosed, each salamander was housed individually into terrariums made with transparent and micro-perforated plastic boxes (11.5 x 11.5 x 6 cm), with coconut litter and gravel as substrates, a plastic cap containing dechlorinated water, and a piece of cork as shelter. Metamorphosed juveniles were fed *ad libitum* with crickets (*Acheta domestica*) and mealworms (*Tenebrio Molitor*).

Ethical note

Field collection of salamanders, subsequent husbandry, and final release at the collection place have been carried out according to the adequate welfare standards for fire salamanders, and were approved by both national (Italian Ministry of Environment, permit number: 0008275.20-04-2018, ISPRA #23501, 23-03-2018) and local authorities (Regione Campania #0203190, 27-03-2018).

Athletic performance test

A convenient approach to the analysis of athletic performance in animals consists of measuring maximal movement performance in response to a stimulus that is perceived as threatening, usually cues of a predator attack (*e.g.,* Diamond *et al.,* 2019; Freymiller *et al.,* 2019; Baxter-Gilbert *et al.,* 2018). We performed a series of pilot tests on a batch of non-experimental animals (10 individuals) in an attempt to elicit impulsive movements. However, even the more invasive version of these tests (*i.e.,* tail pinching) did not elicit consistent and repeatable responses. Most individuals did not react at all, while a few others jumped, u-turned, or sprinted, and the responses were not repeatable over multiple trials. Accordingly, we moved to test individual response to another common threat in fire salamander environments: falling into the water. As mentioned above, adult fire salamanders are fully terrestrial. Accidental falls into the water may be fatal, unless the shore is reached promptly. Preliminary trails revealed a consistent response of salamanders to swimming challenges, which provided us with the basis for setting up an athletic performance test in this species.

Swimming performance was measured three months after the metamorphosis to ensure the performance being representative of a fully terrestrial life stage. Swimming tests were carried out under strictly controlled environmental conditions (room temperature 20°C, humidity 70%; water temperature 15°C) since variability in these conditions might impact individual performance during the test.

The experimental arena consisted of a white circular plastic tank (diameter: 100 cm; water volume: 50L; water depth: 6.5 cm; see Figure 1 B) to avoid possible "blind spots", where individuals may get stuck or have a foothold to climb out of the water. To standardise the starting phase of the test, when the individuals were released in the tank, the centre of the arena was marked with a small plastic circle placed 1 cm above the water surface. Thus, each individual was gently dropped into the water from the centre of this "launchpad", using a humid spoon. Test duration was set to 2 minutes. At the end of the test, individuals were weighted with a precision analytical scales (±0.001g) to calculate the acceleration force. All individuals were fasted 48 h before the tests.

Swimming performance tests were video recorded at high frame rates (120 fps), using a GoPro Hero5 camera (at 1080p) placed above the centre of the experimental arena. Videos were then analysed with the software Tracker 5.1.3 (https://physlets.org/tracker). This software allows characterising physical properties of animal movements using automatic point-mass tracking, provided that sufficiently high-quality videos are used as input (*i.e.,* videos with enough contrast between tracked objects and the background), together with a measure of the body mass. We used the tip of the salamander snout as a point mass for tracking. Tracker allows extracting a wide range of performance variables. For illustrative purposes, here, we provide maximum and average values of velocity and acceleration. Potential errors in automatic tracking were checked by eye for all the individuals analysed by the same operator.

3.3 Results and discussion

All the individuals analysed started swimming immediately after the dive, and continued swimming on the water surface until they reached the edge of the tank (Figure 1 C; Table 1). This behavioural pattern has at least two highly desirable features when testing athletic performances: 1) complete inter-individual consistency; 2) a bi-axial configuration of the entire athletic movement. Consistency of the behavioural pattern is essential, as it implies that individual personality does not modulate an individual's tendency to perform the athletic task at its best, and so it does not act as a confounding experimental factor (Losos *et al.,* 2002; Irschick, 2003; Careau & Garland, 2012; Astley *et al.,* 2013). This argument receives further support by observing that maximum acceleration values are usually reached at the beginning of the test, which is not expected in case personality (*e.g.,* shy vs bold response to a stressful situation) was a significant factor. On the other hand, the bi-axial configuration of the movement over the water surface allows ignoring the third (*i.e.,* vertical) axis when deriving acceleration and other physical descriptors of the athletic performance, simplifying the experimental setup substantially.

Once individuals reached the edge of the tank, the behavioural pattern changed significantly, towards a heterogeneous alternation of resting at the edge, swimming and floating phases. Moreover, over multiple testing, consistency of the pattern was observed neither among nor within individuals, which qualifies this second segment of the test as useless for our purposes. Noteworthy, it implies that endurance measures cannot reliably be derived from this test. At the same time, however, this diverse mix of multiple phases might reveal the emergence of distinct behavioural types along the second segment of the test. If and to what extent behavioural patterns along this segment (*e.g.,* frequency and/or time budget allocated to each phase) might associate with individual personality traits could be an intriguing subject for future experimental scrutiny, given the inherent difficulties in studying personality traits variation in highly elusive and rather static species.

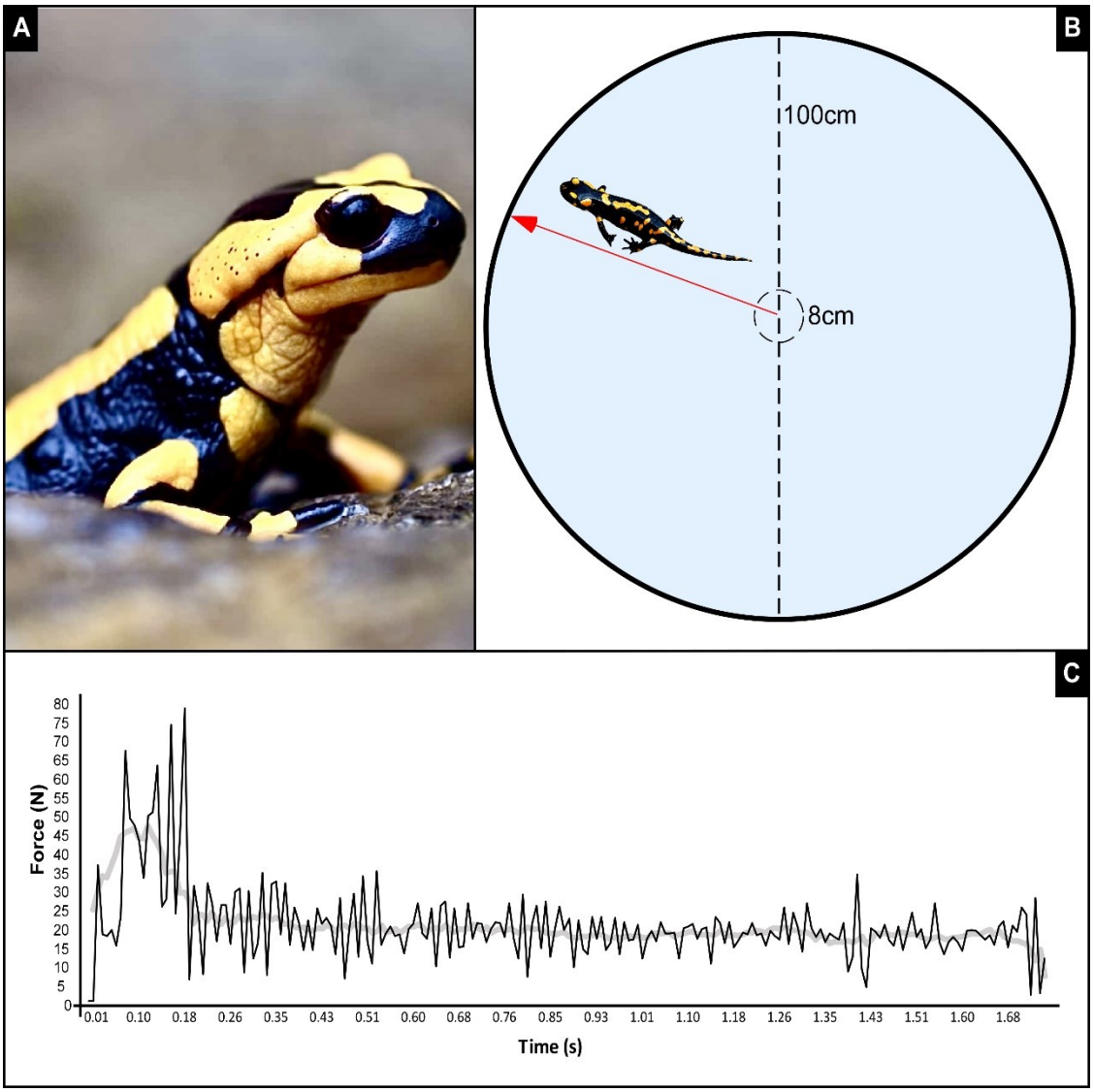
Measuring animals' athletic performances in elusive, slow-moving, or difficult-to-track animals could be very challenging (Biewener & Patek, 2018). Here, we have described a rapid, simple, and low-cost method to measure athletic performance in fire salamanders under standardized, albeit environmentally realistic, laboratory conditions. Importantly, by forcing individuals to perform in the aquatic environment, this method proved successful in avoiding the lack of motivation, in line with results obtained with similar approaches in other organisms (*e.g.,* in rodents, Suvrathan *et al.,* 2010; Can *et al.,* 2012). Finally, a point of significant value of the approach presented here stems from the possibility of gaining comparable athletic performance measures between larval and terrestrial stages. Indeed, when in the aquatic medium, post-metamorphic salamanders show a swimming pattern matching the one used by larvae. In light of this match, our test will allow us to investigate the contribution of carryover effects across life stages to the wide range of processes implicated in the eco-evo-devo of athletic performance in salamanders (Moore & Martin, 2019).

3.4 Tables and figures

Table 1. Maximum (Max) and mean values of speed and acceleration, and mean time to reach the edge of the experimental arena, measured for the post-metamorphic individuals of *Salamandra salamandra* analysed. Values are averages of all tested individuals ± standard error (SE).

Variable	Mean ± SE
Max speed (cm/s)	34.206 ± 1.63
Mean speed (cm/s)	19.185 ± 0.446
Max acceleration force (N)	3949.813± 237.231
Mean acceleration force (N)	1628.205 ± 125.629
Mean time to reach the edge (s)	3.678 ± 0.385

Figure 1. A) The fire salamander *Salamandra salamandra*. B) The arena used for the swimming test (salamander is not in scale). C) Acceleration force profile of an individual as a representative example (black line raw data, grey line moving average).



4. PERFORMANCE AND DEVELOPMENTAL CORRELATES OF RAMPANT MITOCONDRIAL INTROGRESSION IN THE ITALIAN FIRE SALAMANDER

4.1 Introduction

Understanding the processes moulding the geographic patterns of biodiversity is a major task in the ecology and evolutionary research agenda (Canestrelli *et al*., 2016a, b and citations therein). Recently, increasing attention has been paid to the evolutionary processes associated to patterns of mito-nuclear discordance across secondary contact zones (Toews & Brelsford, 2012; Arntzen *et al*., 2016; Harrison & Larson, 2016; Lin *et al*., 2018). Both random and adaptive processes have been proposed to explain instances of differential introgression of mitochondrial vs. nuclear genomes (Toews & Brelsford, 2012).

Results from the study of personality traits variation in the Italian fire salamander *Salamandra salamandra* (see Chapter 2), showed that in this species individuals belonging to alternative mitochondrial lineages spanning a secondary contact zone in central Italy, substantially differ in behavioural traits linked to dispersal (boldness, exploration and activity). Still, the northern mitotype, which introgressed for over 600 km of within the southern nuclear background, is characterized by a slow-thorough dispersal profile, expressed by long decision-making times in an unfamiliar environment and in a cautious exploration and risk-taking attitude. Furthermore, such a behavioural trait difference remains consistent across life stages in the same individuals (*i.e*., larvae and metamorphosed juveniles). This evidence opens for questioning about differences in performance, developmental and physiological traits which usually cluster together with dispersal related behavioural traits, in so-called "dispersal syndromes" (Ronce & Clobert, 2012; Cote *et al*., 2017).

Here, we explored the hypothesis that the two mitochondrial lineages of the Italian fire salamanders spanning the secondary contact zone in central Italy could also differ in performance, developmental and physiological traits related to dispersal.

4.2 Material and methods

Sampling and housing

We collected 90 larvae across the secondary contact zone between the two mitochondrial lineages (Bisconti *et al.* 2018) in the Picentini mountains, central Italy. To reduce the probability of kinship, larvae were collected from distinct breeding sites that were few km apart within this area. We monitored the sampling sites since late winter, so larvae were sampled at birth. Samples were transported in plastic bottles containing stream water (2 L) and kept at dark in a cool box until the arrival at the housing facilities of the Tuscia University at the end of the day. Larvae and metamorphosed juveniles were housed according to the protocol previously described in Chapter 2, under standard conditions (water temperature 10-12° C, room temperature 18-20° C, humidity 65-70%, natural photoperiod), with the permission for temporarily housing at the University facilities granted by the Local Health and Veterinary Centre (A.S.L. Tarquinia, license 050VT427). All procedures followed the relevant guidelines and regulations for welfare and were approved at national (the Italian Ministry of Environment, permit number: 0008275.20-04-2018; the Institute for Environmental Protection and Research, permit number: #23501, 23-03-2018) and local level by competent authorities (Regione Campania #0203190, 27-03-2018).

Genotyping

To identify the mitochondrial lineage of each individual (i.e., the southern or northern lineage of Bisconti *et al*., 2018) we used the protocols previously described in Chapter 2. Briefly, we collected biological tissue for DNA extraction using skin-swabs. DNA extraction was performed using the standard CTAB method. Then, we used polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP) diagnostic technique to assess the alternative mtDNA haplotypes. To avoid any kind of bias, DNA collection and genotyping were performed at the end of all the tests.

Performance traits

Maximal locomotor performances are often used as a proxy for fitness, survival and dispersal (Husak, 2006; Husak *et al.,* 2016; Denton *et al*., 2017), as its speed and acceleration determine the intensity of locomotory activity during these ecological tasks, the time it takes to complete them, and thus their rate, distance and success. Greater performers have been reported at the front edge of expanding populations (Phillips *et al*., 2006, 2010). Accordingly, we measured the same locomotor performances in both larvae and juveniles: swimming. This kind of performances is common for both life stages as fire salamander have aquatic larvae and terrestrial adults which can fall into the water of small brooks and swim to get the pond bound (Lanza *et al*., 2009; Sparreboom, 2014).

Larvae were tested after one week of acclimatization upon arrival at the housing facilities, whereas juveniles were tested exactly three months after the metamorphosis. All individuals were fasted 48 h before the tests. During the tests the room temperature was kept at 20° C and humidity was kept at 70%. Each performance test was recorded using digital cameras (Panasonic DMC-FZ300 camera) and videos were analysed with the software Tracker 5.1.3 (https://physlets.org/tracker/), using the tip of the salamander snout as point-mass for tracking. Then, individuals were weighted with a precision analytical balance (± 0.001 g), in order to calculate the swimming force. We used the maximal velocity and swimming force along with the time to reach the end of the arena as a measure of maximal locomotor effort.

(i) Swim test on larvae. Locomotor performance of larvae was measured by forcing larvae to swim. The experimental arena consisted of a transparent plexiglass tube (diameter 3 cm x length 1 m) placed in vertical position filled with dechlorinated water (12° C) and closed at one end with a silicone cap. Each individual was gently inserted into the water from the top of the tube using a humid spoon. Swimming performance tests were video-recorded at high frame rates (120 fps), placed in front of the experimental arena. Video were visually inspected by the same operator, using 5 frames step size.

(ii) Swim test on juveniles. To measure locomotor performance in post-metamorphic juveniles we used the protocol of de Rysky *et al*., (2021b; see Chapter 3). Each individual was gently dropped into the circular arena filled with dechlorinated water (15° C) using a humid spoon. The test finished once each individual reached the edge of the arena. Video were analysed using the automatic point-mass tracking and one frame as step size. Potential errors in automatic tracking were checked by eye for all the individuals analysed, by the same operator.

Physiological proxies

(i) Heart rate. The heart rate measurements after locomotory efforts can be a proxy for metabolic cost of effort, resulting as indirect indicator of energy expenditure for physical exercises (Green, 2011; Aubret *et al.,* 2013; Catenazzi, 2016). We measured the heart rate (heart beats/min) of larvae immediately after the swimming performance test, transferring each individual into a glass chamber. The heart rate was then recorded by using a digital microscope (Dino Lite) set above the glass chamber.

(ii) Morphometric traits. We measured the body condition index (hereafter BC), the snout-vent length (here after SVL) and the growth rate of both larvae and juveniles. Measurements were taken at the arrival at the housing facilities and after 6 weeks in larvae, and immediately after the metamorphosis and after 3 months in juveniles. Larvae were set in a glass chamber containing dechlorinated soft water, then photographed by using a digital microscope (Dino Lite) and subsequently weighted by using an analytical balance. Juveniles were placed in a petri dish and photographed by using a digital camera (Panasonic DMC-FZ300), then weighted. A 1 mm grid paper was used as reference scale. The SVL measures have been taken three times from photographs by using digital image analysis software Image J (Mott *et al.* 2010; Sanchez *et al.* 2018). Then, the mean was used to reduce errors. The BC was calculated according to the method of Baker, (1992). Relative growth rate was obtained as SVL increment relative to the initial individual length.

(iii) Larval period duration. Development rate in terms of time to metamorphosis can affect many performances of both larval and metamorphic life-stages (carry-over and latent effects) and it has been correlated with fitness (Wilbur & Collins 1973; Alcobendas *et al.,* 2004; Ficetola & De Bernardi, 2006). We measured the larval period duration (days) as the number of days from the arrival at the housing facility to metamorphosis under standard conditions,

Statistical analysis

To test the effect of mitotype (fixed factor) we ran a series of linear mixed models (LMMs; factorial, sums of squares, type III, confidence interval 95%). Each variable was entered singly for each life-stage. The models for performances traits, heart rate and larval period duration were fitted with the maximum likelihood (ML) method. The same LMMs were used for morphometric traits, including subjects as random effect and time as additional within

subject fixed factor (two levels for both larvae and for metamorphosed juveniles: arrival-6 weeks and metamorphosis-3 months respectively). These latter models were fitted with the restricted maximum likelihood (REML) method. To test for consistency of mitotype effect across life-stages (larval and metamorphosed) on performance and morphological traits we ran the same LMMs for repeated measures including life-stage as additional within subject fixed factor. Finally, we evaluated the relationships between traits both within and between life-stages using the Spearman rank correlation test (confidence interval 95%). All the analysis were performed using SPSS v.23. The graphs were drawn using the software Canvas 11 (ACD Systems of America, Inc.).

4.3 Results

According to the PCR-RFLP diagnostic, 62 out of 90 individuals showed northern lineage mitotype, and 28 showed the southern lineage mitotype.

Performance traits and physiological proxies

Results of the analysis on the performance traits are reported in Tabs. 1, 2 and Figure 1. We found no difference between the alternative mitotypes in the measured performance traits. Larvae of the northern mitotype had higher heart rate after locomotory effort and had higher BC and SVL both at the arrival and after 6 weeks compared to the southern ones. BC and SVL of larvae did not change differently between genotypes because no significant effect of mitotype x time interaction was evident for both morphometric measures. However, the difference in SVL between northern larvae and southern ones was higher at 6 weeks. No difference between mitotypes was found in the relative growth rate between the arrival and 6 weeks, as well as in the larval period duration.

No difference between mitotypes was found in both BC and SVL of juveniles, and they did not change differently overtime between genotypes because no significant effect of mitotype x time interaction was evident. However, individuals of the southern mitotype had higher relative growth rate between metamorphosis and 3 months than the northern one. Results of the analysis on the physiological proxies are reported in Table 1 and Figures 2, 3.

Consistency of mitotype effect across life-stages

The absence of differences in performance traits was still present across life-stages. However, the northern mitotype had lower maximal speed and swimming force than the northern ones at larval stage, while had higher maximal speed and swimming force compared to the southern ones once metamorphosed. The northern mitotype had higher BC compared to the southern one both at larval and metamorphosed stage, and the difference in BC between northern and southern mitotypes was higher in the larval stage than in the juvenile one. No differences between mitotypes were found in the SVL, but mean values differed more at larval stage. No difference was found between mitotypes in the relative growth rate, but it was higher at larval stage. The results of the analysis across life-stages are reported in Table 2.

The correlation analyses between traits showed the BC of larvae at the arrival negatively related to the BC of juveniles at the metamorphosis ($rS = -0.256$, $p = 0.023$). Also, the larval period duration was negatively related to the BC of larvae at 6 weeks ($rS = -0.377$, $p = 0.003$). We found the BC and SVL of juveniles at the metamorphosis positively related to the BC and SVL of larvae at 6 weeks ($rS = 0.372$, $p = 0.004$ and $rS = 0.404$, $p = 0.002$ respectively), as well as to the SVL of juveniles at 3 months ($rS = 0.386$, $p = 0.001$). Also, the SVL of juveniles at 3 months was positively related to the SVL of larvae at 6 weeks and to the relative growth rate of juveniles ($rS = 0.485$, $p = 0.000$ and $rS = 0.442$, $p = 0.000$ respectively).

4.4 Conclusions

We found that alternative mitotypes of the Italian fire salamander spanning the secondary contact zone in central Italy do not differ in locomotor performances, yet they showed marked differences in physiological traits. In fact, the northern and the southern mitotypes performed similarly at both larval and metamorphosed stage under the same swimming challenge, but with different efforts and metabolic costs, coupled with significant differences in body condition and body size at the larval stage.

Interindividual variations in athletic performances have important eco-evolutionary implications, as they can contribute to driving the evolution of morphological, physiological, and behavioural traits in the context of energetic and environmental constraints (Irschick & Higham, 2016; Biewener & Patek, 2018). Locomotor performances are functionally linked to many life-history traits, and the energy production and allocation are crucial given the high energy costs of these abilities, thus, physiological, behavioural and performances traits are functionally related to each other by a common underlying constraint (Careau *et al*., 2008; Careau & Garland, 2012; Martin *et al*., 2015). Consequently, given that mitochondria are either the producer and the manager of energetic and oxidative metabolism (see Chapter 1.4), and thus of the constraints in the organism performance, a functional link between mitochondrial genotype variation and the variations in locomotor capacity, metabolism or development rates, results particularly plausible. We did not find a significant effect of the mitotype on the maximal locomotor capacity in fire salamander larvae and juveniles, but we found differences in the effort to get such locomotor ability. This results in alternative strategies with different potential outcomes in term of fitness. Further investigations should be focused on characterizing the variation in terms of fecundity, survival rates at metamorphosis and recruitment. Indeed, differences in the recruitment rates among mitochondrial lineages co-occurring in a secondary contact zone may result in unbalanced demographic proportion at reproduction, and thus differential introgression of the two lineages (cfr. Toews & Brelsford, 2012; Petit & Excoffier, 2009; Canestrelli *et al*., 2016a),

Finally, the difference in locomotor performance between the life stages suggests ontogenetic changes in locomotor performances' evolution. These differences could be mainly linked to variation in morphology (e.g., tail-leg shift) and physiology (e.g., energy allocation), thus differently predict survival across life-stages. On the other hand, we found that the mitotype significantly affected the metabolic cost of effort in larvae and morphometric traits of both larvae and juveniles, suggesting different life-history syndromes between the two mitotypes of the fire salamander. In particular, the larvae of the northern mitotype were fatter and bigger than the southern ones and had a higher metabolic cost of effort, indicating a slow life history profile accordingly to the allometry of performance and the energy allocation/compensation model (Steyermark, 2002; Nilsson, 2002; Glazier, 2005; White & Kearney, 2013). Bigger individuals have a lower basal metabolic rate and allocate less energy on activity, resulting in higher metabolic cost of effort (Lovegrove, 2000; Lailvaux & Husak 2017; Kozłowski *et al.,* 2020), but more investment in growth, reproduction and slow/long-distance dispersal (Kaitala, 1999; Blackmer *et al.,* 2005; Careau *et al.,* 2009; Careau & Garland, 2012; Louppe *et al.,* 2018). The consistency of higher body condition of the northern lineage across life-stages underpins a slow profile in the introgressed northern mitotype that could confer survival and reproductive advantages in adulthood (Stamps, 2007; Lowe, 2010; Vetter *et al.,* 2016; but see Smith & Blumstein 2008), given the carry-over effects of early life-stages (John-Alder & Morin, 1990; Beck & Congdon, 2000; Chelgren *et al.,* 2006; Searcy *et al.,* 2014; Krause & Caspers, 2016; Moore & Martin, 2019).

Our results on the locomotor performance do not follow the marked differences in behaviours between the two

mitotypes of fire salamander reported in the previous Chapter 2, yet they support the hypothesis that alternative mitotypes could carry specific, potentially adaptive, life-history traits, allowing their permeation irrespective of the nuclear genome across a secondary contact zone. In particular, in the case of the fire salamander of central Italy, a slow life-history strategy could have driven the rampant mitochondrial expansion of the northern lineage into the nuclear genome background of southern.

4.5 Tables and figures

Table 1. Outcomes of the LMMs for performance traits and physiological proxies overtime in larvae and juveniles. BC = body condition. SVL = snout-vent length. F = fixed effects estimates. p values (p) associated with the confidence interval of 95% are reported. Significant values concerning genotype effect ($p < 0.005$) are highlighted in bold.

	Life-stage					
	Larvae			**Juveniles**		
Variables	**Effect**	**F**	**p**	**Effect**	**F**	**p**
Swim test						
Max speed (cm/s)	mitotype	0.267	0.607	mitotype	0.349	0.556
Max acceleration force (N)	mitotype	0.129	0.720	mitotype	0.975	0.327
Time to reach the end of the arena (s)	mitotype	0.554	0.459	mitotype	0.103	0.749
Physiological proxies						
BC	mitotype	8.462	**0.005**	mitotype	3.594	0.062
	time	0.406	0.526	time	1.321	0.254
	mitotype x time	0.155	0.695	mitotype x time	0.330	0.567
SVL (cm)	mitotype	8.019	**0.006**	mitotype	0.541	0.464
	time	732.983	**0.000**	time	596.288	**0.000**
	mitotype x time	1.343	0.252	mitotype x time	2.947	0.090
Relative growth rate (cm)	mitotype	0.050	0.825	mitotype	4.059	**0.048**
Heart rate (heart beats/min)	mitotype	9.338	**0.003**			
Larval period duration (days)	mitotype	1.331	0.252			

Table 2. Outcomes of the LMMs for performance traits and physiological proxies across life stages. BC = body condition. SVL = snout-vent length. F = fixed effects estimates. p values (p) associated with the confidence interval of 95% are reported. Significant values concerning genotype effect ($p < 0.005$) are highlighted in bold.

Variable	Effect	F	p
Swim test			
Max speed (cm/s)	mitotype	0.018	0.894
	life-stage	11.681	0.001
	mitotype x life-stage	0.497	0.484
Max acceleration force (N)	mitotype	1.086	0.300
	life-stage	336.693	0.000
	mitotype x life-stage	1.080	0.301
Time to reach the end of the arena (s)	mitotype	0.410	0.524
	life-stage	0.029	0.865
	mitotype x life-stage	0.059	0.809
Physiological proxies			
BC	mitotype	14.102	**0.001**
	life-stage	1.878	0.177
	mitotype x life-stage	12.960	0.001
SVL (cm)	mitotype	2.337	0.253
	life-stage	795.820	0.000
	mitotype x life-stage	1.428	0.257
Relative growth rate (cm)	mitotype	1.339	0.253
	life-stage	9.361	0.004
	mitotype x life-stage	2.021	0.165

Figure 1. Performance traits of fire salamander larvae and juveniles. (A) Maximal speed; (B) Maximal acceleration; (C) Time to reach the end of the arena. Values are means per mitotype ± standard error (SE).

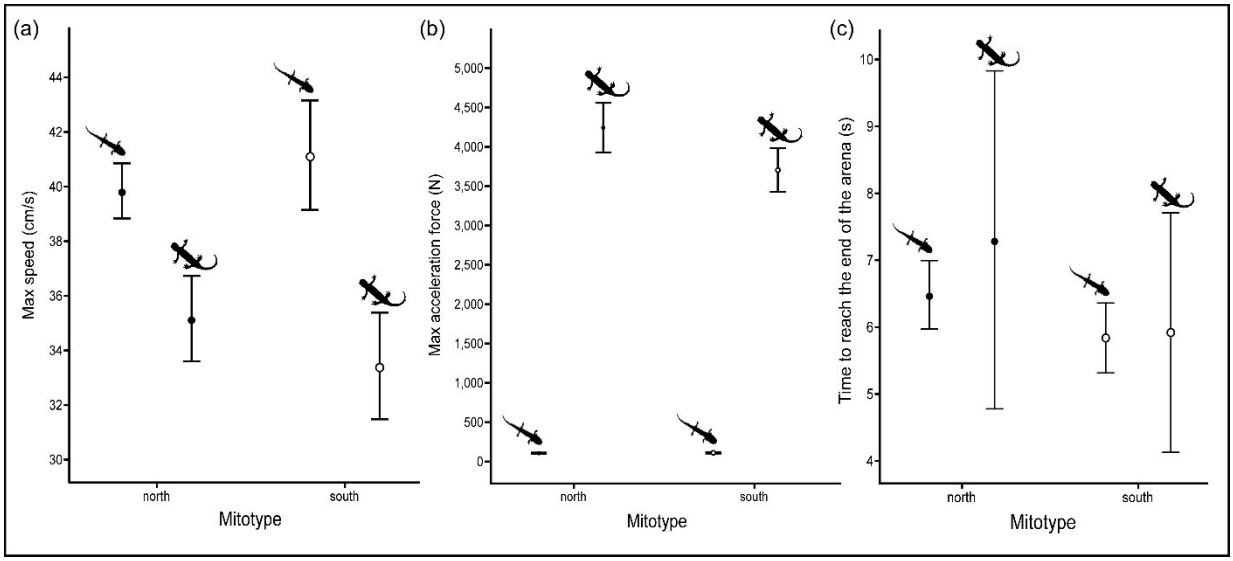


Figure 2. Physiological proxies in fire salamander larvae. (a) Heart rate of larvae after swimming effort; (b) Larval period duration. Values are means per mitotype ± standard error (SE).

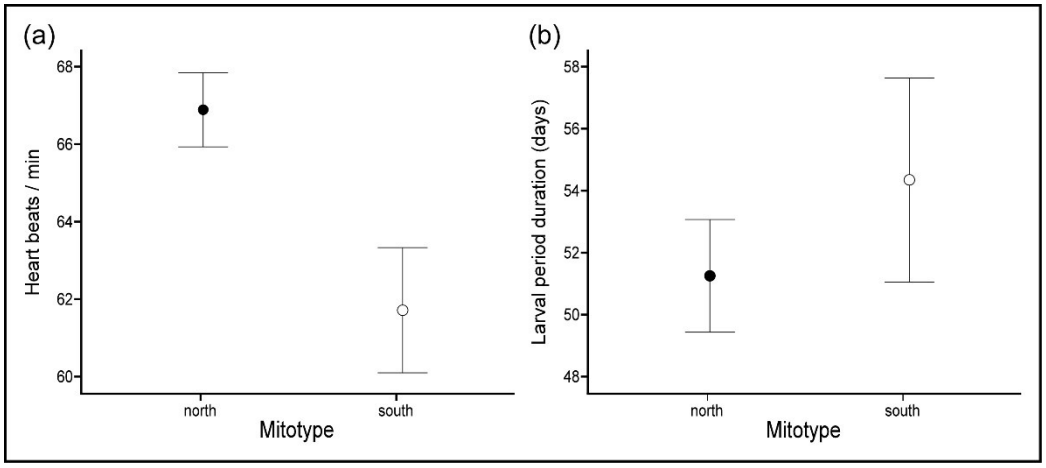
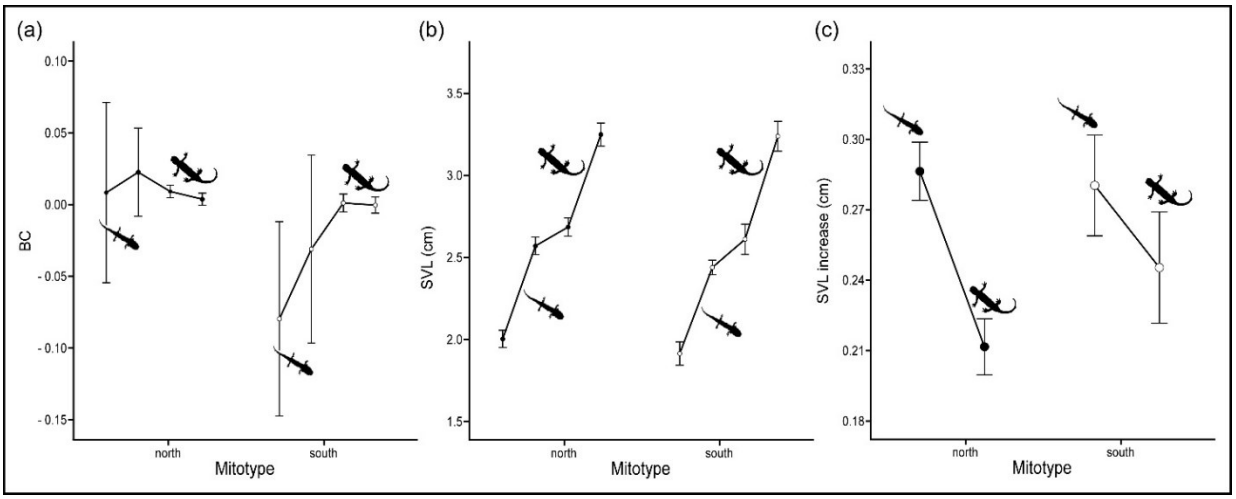


Figure 3. Ontogenetic profiles of morphological traits in fire salamander larvae and juveniles. (a) Body condition (BC); (b) Snout-vent length (SVL). The four temporal points in both BC and SVL profiles represent arrival, 6 weeks, metamorphosis, 3 months; (c) Relative growth rate expressed as snout-vent length (SVL) increase. The two temporal points in the SVL increase represent arrival-6 weeks and metamorphosis-3 months. Values are means per mitotype ± standard error (SE).



5. DISCUSSION

5.1 *Mitochondrial genotype affects behavioural and life history traits linked to dispersal in Salamandra salamandra*

The links between genotypic and phenotypic polymorphisms can reveal mechanisms behind spatial and temporal patterns of variations in animal biodiversity (Storz & Wheat, 2010). However, it could be challenging to characterize the nature of these variations, because little genetic changes can result in complex phenotypic changes, while substantial genetic changes may not influence the phenotype at all (Ghiselli & Milani, 2019). Patterns of discordances between mito-nuclear genotypes, especially mitochondrial introgression, represent a useful opportunity for investigating the functional, adaptive and eco-evolutionary significance of variations at mitochondrial loci (see Chapter 1.1, 2 and 3). This research provides experimental evidence of different behavioural and life-history traits linked to dispersal in two mitotypes of the Italian fire salamander *S. salamandra*. In the next paragraphs we outline the rationale behind this link in our data.

MtDNA has long been used in phylogenetic and biogeographic studies to reconstruct historical range dynamics, inferring the evolutionary and demographic past of populations and species (Avise *et al.,* 1987; Galtier *et al.,* 2009). In particular, the mitochondrial cytochrome b (cyt-b) and cytochrome c oxidase I (COI) are genes widely used in systematic studies to explore divergences at many taxonomic levels because they have both a slow and rapid rate of molecular evolution, as well as both conservative and variable regions or domains (Farias *et al.,* 2001; Hebert *et al.,* 2003). The cyt-b and COI genes are involved in the synthesis of adenosine triphosphate by oxidative phosphorylation, the mitochondrial respiratory chain (*i.e.,* respiratory electron transport), the heat production by uncoupling proteins and in the cardiac muscle contraction (Esposti *et al.,* 1993; Wilson & Vinogradov, 2014). The energy and redox balance are both considered as central eco-evolutionary constraints determining life-history traits and their trade-offs (Dowling & Simmons, 2009; Costantini *et al.,* 2010; Salin *et al.,* 2015), resulting to be the mechanistic link to ecologically and energetically expensive tasks such as dispersal.

Metabolism has been proposed as the key-link between the biology of individual organisms and the eco-evolutionary dynamics at population, community and ecosystem level (Brown *et al.,* 2004; van der Meer, 2006; Glazier, 2014; Burger *et al.,* 2019), and many studies showed that metabolism affects POLS traits including behaviour (Martins *et al.,* 2011; Mathot & Dingemanse, 2015; Rittschof, 2015; White *et al.,* 2016; Salzman *et al.,* 2018). In particular, two main models have been formulated to explain the mechanistic relationship between personality and energy metabolism: the increased intake model and the compensation model (Careau *et al.,* 2008; Careau & Garland, 2012). The increased intake model predicts a positive association between energetically demanding behaviours and metabolic rates, leading proactive, explorative and bold individuals to have high metabolic rate with increased energy outputs and a reduced metabolic cost of effort (Nilsson, 2002; Biro & Stamps, 2010). The compensation model predicts a negative association between energetically demanding behaviours and energetic output instead, leading active, explorative and bold individuals to compensate for their higher energy consumption in activity at the expense of other life history traits (Steyermark, 2002; Blackmer *et al.,* 2005; Vaanholt *et al.,* 2007). This is because according to the allocation principle, limited energetic resources and processing impose trade-offs between traits (Wiersma *et al.,* 2007; Careau & Garland, 2012; Salzman *et al.,* 2018; Biro *et al.,* 2020). Therefore, lower energy investment in energetically demanding behaviours and performances such as exploration, resulting in shy and less active/explorative behaviours, can lead to increased growth, fitness or lifespan and *vice*

versa (Metcalfe & Monaghan, 2003; Stamps, 2007; Careau *et al.,* 2008, 2009; Réale *et al.,* 2010; Louppe *et al.,* 2018; Adriaenssens & Johnsson, 2011; Hoogenboom *et al.,* 2012).

The behavioural and life history profiles in both larval and metamorphosed juveniles of fire salamander that emerged in this research are in accordance with the compensation model, resulting in different dispersal strategies. In fact, the northern mitotype of the *S. s. salamandra* allocates more energy for growth than for activity, showing higher body size and condition, higher metabolic cost of effort, shyness, less active and explorative behaviour. Conversely, the southern mitotype of *S. s. gigliolii* allocates more energy for activity than for growth, showing lower body size and condition, lower metabolic cost of effort, boldness, more active and explorative behaviour. Consequently, our results suggest that the northern mitotype of *S. s. salamandra* can be placed in the slow lane of life history syndromes with a slow-thorough dispersal strategy, which can increase survival, longevity and reproduction. On the other hand, the southern mitotype of *S. s. gigliolii* has fast life history syndromes with a fast-superficial dispersal strategy, which can reduce survival and longevity due to higher exposure to predators or disease, with consequent lower reproduction.

These patterns of big-shy-less explorative traits *versus* small-bold-more explorative ones are also in accordance with the allometric theory of metabolism, which scales the metabolic rate with the body size (Glazier, 2005; Kozłowski *et al.,* 2020), as well as for behaviour and life history traits (Dial *et al.,* 2008; Polverino *et al.,* 2016; Kelleher *et al.,* 2017). Accordingly, bigger individuals have slower metabolism, lower self-maintenance costs and more energy for growth, reproduction and dispersal (Kaitala, 1999; Lovegrove, 2000; Blackmer *et al.,* 2005; Speakman, 2005; Careau *et al.,* 2009; Careau & Garland, 2012). The evolutionary causes of the allometric relationship between metabolic rate and body size is even underpinned by the genetic associations between traits that contributes to metabolic rate (Glazier, 2005; Sadowska *et al.,* 2009; Zub *et al.,* 2012), emphasising the link between mitochondria and dispersal phenotypes (Careau *et al.,* 2011; Rollins *et al.,* 2016). In fact, we found that the mitotype has significant effect on the behavioural, morphological and physiological traits related to dispersal in the Italian fire salamander, indicating that the differences between the two subspecies *S. s. Salamandra* and *S. s. giglioli* are due to the mitochondrial genotype. This is also supported by the insights on the absence of possible nuclear structure (see Chapter 2).

As we have attempted to illustrate here, bioenergetics provides the link between mitochondrial genotype and dispersal phenotype, as mitochondrial genetic variations can affect dispersal traits like behaviour, locomotory performances, metabolic cost of effort and other related life history traits (*i.e.,* growth rate, body size and condition, life-stage duration, sexual maturation). Indeed, the studies showing the repeatability of metabolic rate and its consistency across time and contexts (Nespolo & Franco, 2007) along with studies showing the additive genetic variance (heritability) in metabolic rate (Nespolo, 2007; Rønning *et al.,* 2007; Tieleman *et al.,* 2009; but see Bacigalupe *et al.,* 2004), and the mito-nuclear pleiotropic and epistatic effects on energetic and oxidative state (see Chapter 1.4), strongly suggest a complex genetic architecture of metabolic rate (Arnqvist *et al.,* 2010; Konarzewski & Książek, 2013), with a key role of mitochondrial genotype (Pichaud *et al.,* 2012).

Given the metabolic consequences of variation in the mitochondrial genome that influence animal dispersal, empirical studies on the mitochondrial genetic effects at both behavioural and physiological level result crucial to test whether mtDNA carries specific phenotypic features with important evolutionary implications. This research contributes to better understand the eco-evolutionary implication of mitochondrial genetic variation, showing the existence of different dispersal mitotypes.

5.2 *A new method to measure locomotor performance in the fire salamander*

Organisms are always engaged in a wide range of performances, even during inactivity, thus the associated variation in energy expenditure is a key link between performances and overall fitness. As mentioned in the previous chapters, locomotor performance is crucial for dispersal processes, as its speed and acceleration can determine dispersal rate, distance and success. Measuring maximal locomotor performances allow to understand their role in many eco-evolutionary processes such as dispersal, thus studying individual differences in maximal locomotor capacity is an important step toward understanding how species differ in dispersal behaviours (Fronhofer *et al.,* 2015; see Chapter 3 and 4) and how these differences can produce biogeographic and genetic patterns (Lowe & McPeek, 2014). However, it can be challenging in elusive and slow living animals such as the fire salamander (see Chapter 4). We provided a simple, rapid and low-cost test to characterize the maximal locomotor performances in the fire salamander *S. salamandra*, which showed remarkable dispersal capacities in spite of its apparent paucity of movement (see Chapter 4). In particular, our method allows to measure the maximal performance (*i.e.,* speed and acceleration) avoiding the potential problems with measuring this performance in the lab, such as the standardization of *stimuli* and psychological factors (*i.e.,* motivation and defensive behaviours). Indeed, measuring maximal locomotor performances under artificial conditions often implies the use of manual stimulation (*i.e.,* manipulation, pinching, tapping or pressing) and unfamiliar environments, which can cause not only problems of *stimuli* standardization, but may also not be enough to stimulate the animals to move, or may induce different reactions, such as lack of motivation to perform and/or the display of extreme defensive behaviours instead of moving. In this case there is the risk of measuring "behaviour" rather than "performance" (Careau & Garland, 2012), and the measured performance may also not reflect what animals do under natural circumstances (Irschick, 2003; Astley *et al.,* 2013).

In nature, motivation is likely to vary from situation to situation, leading to behavioural modulation of the effort put into performances, thus behaviour can act as a filter between performance capacity, which is physically determined, and realised performance, which has direct fitness and survival consequences (Irschick, 2003; Irschick & Garland, 2001; Husak, 2006; see Chapter 4). Consequently, laboratory speed may not be representative of the maximum capacity that animals use, which is likely to occur under high-threat conditions and risky situations, such as during escape in the field, when they capture prey or during territorial contests as these may be contexts in which maximal speed is subjected to selection (the model of locomotor compensation, Irschick, 2003; Irschick *et al.,* 2005b; Husak, 2006; Husak & Fox, 2006). Similarly, forced performance tests in the laboratory may approximate maximal performance, and therefore reflect physiological capacities, not giving individuals the choice of performing at submaximal levels (Careau & Garland, 2012). In fact, forced performance tests are commonly used in animals to test both behavioural or biomolecular responses to stress or drugs (*i.e.,* forced swim test in rodents, Porsolt *et al*., 1978; Can *et al.,* 2012), and to evaluate endurance (*i.e.,* treadmills for terrestrial organisms, Dougherty *et al.,* 2016; Hudson *et al.,* 2016b; Denton *et al.,* 2017). Our test imposed an extreme situation for terrestrial organisms represented by the aquatic environment, which thus forced them to perform at their best swimming to survive.

Furthermore, our test would allow to compare performance between larvae and juveniles of the fire salamanders, which can be crucial for evaluating both the contribution of each stage to dispersal and the consistency of performance capacity across life-stages. Indeed, studies on the effects of ontogenetic changes on the evolution of locomotor performances in lizards, snakes, butterfly and fish, reported significant changes in maximal sprint speed

capacity between hatchlings, juveniles and adults, that differently predict survival across life-stages (Arnold, 1983; Garland, 1985; Wassersug & Sperry, 1977; Huey, 1980; Watkins, 1997; Wilson & Franklin, 2000), while others explore the carry-over effects of early life-stages (John-Alder & Morin 1990; Beck & Congdon 2000; Chelgren *et al.,* 2006; Searcy *et al.,* 2014; Krause & Caspers 2016; Moore & Martin, 2019).The differences can be mainly linked to variation in both morphological and physiological traits (*i.e.,* tail-leg shift, energy allocation; Arnold, 1983; Garland, 1985), stressing the relationship between variation in locomotory performance and physiological correlates that shape life-history strategies (Garland, 1984; Bennett *et al.,* 1984).

5.3 *The "slow-thorough" dispersal mitotype as potential driver of mitochondrial introgression*

Despite the growing literature on dispersal processes (see introduction, section 1.2), our knowledge of the link between genetic and phenotypic variations of dispersal traits is scanty. However, is noteworthy that geographic patterns of genetic variation within contact zones can promote and be promoted by spatial variation in dispersal traits (Canestrelli *et al.,* 2016a, b). Fast dispersal profiles have been widely reported at the range-edge of expanding populations, suggesting a combination of spatial sorting and natural selection of fast dispersal traits for a successful invasion/colonisation (Phillips *et al.,* 2010; Shine *et al.,* 2011; Chuang & Peterson, 2016). Nevertheless, this is not the case of fire salamander in the Peninsular Italy. Here, we showed that the expanding mitotype of this species has a slow-thorough dispersal strategy coupled with a slow life history syndrome, providing experimental evidence that mitochondrial genotype brings phenotypic traits linked to dispersal. Moreover, our results suggest that these dispersal characteristics of the introgressed mitotype could be drivers of its introgression. In this section we describe the advantages of these slow phenotypic profiles outlying how they could promote/be promoted by mitochondrial introgression.

As opposed to fast individuals, slow individuals are characterized by slow metabolic rate and activity levels, larger body sizes, shy personality and less explorative attitudes, and show longer lifespan (see introduction, section 1.4 and Chapter 2; but see Polverino *et al.,* 2018). These characteristics allow individuals to have lower self-maintenance costs and therefore they could allocate resource for growth, reproduction and dispersal, according to the compensation hypothesis (see Chapter 4.1). Body size and condition have a lot of influence on dispersal, as they can affect boldness, foraging and explorative activity (Polverino *et al.,* 2016 and citation therein). The acquisition of a good and robust physical condition is crucial in determining the extent to which animals disperse, given that long-distance movements are energetically expensive and can involve a high risk of mortality (Barbraud *et al.,* 2003; Bowler & Benton 2005; Stevens *et al.,* 2012), imposing a trade-off between growth and mortality (Stamps, 2007). Indeed, bigger individuals with sufficient fat reserves (*i.e.,* better body condition) have higher dispersal propensity and capacity, due to enhanced locomotor performances (*i.e.,* speed and endurance) and reduced cost of locomotion that allow to travel longer distances (Jenkins *et al.,* 2007; Bonte & De La Peña, 2009; Whitmee & Orme, 2013; Anholt, 1990; Belthoff & Dufty, 1998; Hedenström, 2003; Cabrera-Guzmán *et al.,* 2013).

Dispersal of bigger and shy individuals can be density-induced due to the effect of interspecific crowding, in particular for communal breeding amphibians, as synchronized metamorphosis occurs, and adult stages are territorial and philopatric. In fact, bigger and shy larvae might be the more dispersive as they are more likely to drift away to avoid conspecific competitions when their retreats become too small to provide an adequate food (Thiesmeier & Schuhmacher, 1990), given the ponds' limited space and food where cannibalism may occur (Degani, 1993; Reques

& Tejedo, 1996; Sadeh, 2012; Limongi *et al.,* 2014; Manenti *et al.,* 2015). Furthermore, bigger larvae have greater escape performances (Fitzpatrick *et al.,* 2003), enhancing their survival probability. The body size and condition at larval stage can also affect larval period duration and body size and condition at metamorphosis, influencing post-metamorphic survival and movement patterns (Chelgren *et al.,* 2006; Earl & Whiteman, 2015; Burraco *et al.,* 2017; Ousterhout & Semlitsch, 2018; see Chapter 3). Metamorphosis at higher body size and condition can confer an advantage for metamorphosed juveniles in competitions with both conspecifics and heterospecifics, as well as increase juveniles and adult's performances, dispersal and survival (Werner, 1986; Semlitsch *et al.,* 1988; Beck & Congdon, 2000; Altwegg & Reyer, 2003; Cabrera-Guzmán *et al.,* 2013; Searcy *et al.,* 2014; Rasmussen & Rudolf, 2015; Yagi & Green, 2017; but see Ficetola & De Bernardi, 2006). Consequently, it is not surprising that bigger and fatter individuals display greater survival in new patches (Gundersen *et al.,* 2002), resulting to be the most fecund founders (Bonte & De La Peña, 2009). Indeed, bigger individuals might have increased survival just because their shy, less active and fearful personality, as these traits could compensate their higher detectability by predators due to their larger size (Best *et al.,* 2020; but see Ward-Fear *et al.,* 2018). In the case of fire salamander, given the increased exposure of juveniles to predators near ponds, as they lack defensive strategies, a predation risk-induced dispersal may favour less active, shy and cautious bigger individuals. This is because they escape from predators, resulting to be more dispersive, while fast and bolder individuals might ignore predators, as they use less energy to surveillance (Cote *et al.,* 2010a; Jones & Godin, 2010; but see Laakkonen & Hirvonen, 2007). Conversely, they are good at hiding, as they use more the shelter and are less active, thus reducing the encounter rate with predators and increasing their survival (Sih, 1997; López *et al.,* 2005; Roznik & Johnson, 2009; Carter *et al.,* 2010; Hulthén *et al.,* 2017). Furthermore, the higher use of shelter can also reduce the risk of dehydration, further increasing their survival (Rothermel & Luhring, 2005; Roznik & Johnson, 2009).

Physiological and behavioural fast-slow/bold-shy syndromes are associated with proactive-reactive coping strategies/styles, which are defined as repeatable, consistent and heritable sets of behavioural and neuroendocrine stress responses to common challenges faced by animals (Koolhaas *et al.,* 1999, 2010; Groothuis & Carere, 2005; Réale *et al.,* 2010). According to this wider definition of syndromes it has been suggested that reactive animals have higher corticosterone responses to stress, which apparently increases vigilant behaviour, leading to slow behavioural responses, neophobia and hesitation to explore, which outline a shy, prudent and fearful personality profile (Koolhaas *et al.,* 1999, 2010; Lewis & Sullivan, 2020). Indeed, corticosterone has also been correlated to dispersal by influencing many traits related to dispersal, such as individual with higher corticosterone levels have slower metabolic rates, higher body condition and locomotor performances, resulting in successful exploration for foraging (Belthoff & Dufty, 1998; Martins *et al.,* 2007; Miles *et al.,* 2007; Liebl & Martin, 2012, 2013; but see Wack *et al.,* 2013). According to the copying style theory, reactive individuals are slow-thorough explorers, as they show higher vigilance and prudent exploration, which may take time to acquire information of surrounding environmental condition, but it results in a more accurate evaluation (Verbeek *et al.,* 1994; Koolhaas *et al.,* 1999, 2010; Groothuis & Carere, 2005; Réale *et al.,* 2010). This more accurate evaluation of the surroundings can result in higher flexibility in response to environmental changes (less routine-like behavioural tendencies, Koolhaas, *et al.,* 1999, 2010; Coppens *et al.,* 2010), an increased probability of finding good feeding grounds, and higher fitness in stressful situations such as the dispersive phase in novel environments (Cockrem, 2005, 2007; Coppens *et al.,* 2010). Many authors suggest that the departure of juveniles overall might not be random (Rothermel, 2004; Zollner & Lima, 2005; Jenkins, *et al.,* 2006; Rittenhouse & Semlitsch, 2006; Patrick, *et al.,* 2007; Timm *et al.,* 2007), leading to the

hypothesis of an informed dispersal (Clobert *et al.,* 2009). However, there is little evidence of the so-called prospecting foray loops as searching strategies to acquire information in amphibians' juveniles compared to other taxa, and currently there is not yet a comprehensive understanding of how juveniles acquire information about the habitat in each dispersal phase (Pittman *et al.,* 2014 and citations therein). This unexplored hypothesis is a critical area for future research in cognitive abilities and dispersal (Delgado *et al.,* 2009, 2010), as it may significantly influence dispersal patterns, as suggested by studies exploring the causes of individual differences in animal exploration and search (Reader, 2015 and citations therein).

The slow-thorough dispersal profile outlined in this study is in accordance with other studies reporting the existence of slow and long-distance dispersal phenotypes (*i.e.,* Myers & Krebs, 1971; Armitage, 1986; Selonen & Hanski, 2006; Von Merten & Siemers, 2012). This suggests the advantages of a slow-thorough dispersal strategy coupled with slow life history syndrome in terms of fitness and survival, as they could minimise the risks and compensate for the uncertainties of reproductive success through high adult survival and future reproduction. Consequently, a positive selection of slow traits behind the invasion front, after the first expansion wave events, could be necessary for successful settlement as it could increase population density at the expanding front with consequent gene flow. Indeed, growing evidence indicates that it might be the coexistence of multiple dispersal phenotypes and their spatial distribution that drives range expansions and their eco-evolution dynamics and impacts (Bowler & Benton, 2005; Duckworth, 2008; Clobert *et al.,* 2009; Cote *et al.,* 2017). From this perspective, the coexistence of fast-slow dispersal and life history strategies is crucial to ensure both the brake of the edge with a fast-superficial exploration, charged by fast individuals at the expansion front, and the subsequent settlement with a slow-thorough dispersal, charged by the slow rear-guards. These slower individuals on the back front might be likely to hybridize with conspecific along the contact zone, allowing the return to equilibrium of dispersal rate and population dimension values (Cobben *et al.,* 2015; Canestrelli *et al.,* 2016b), and leading to the introgression of slow-thorough dispersal mitotypes.

6. CONCLUSION

The case of mitochondrial introgression in the fire salamander *S. salamandra* of Italian peninsula provided the unique opportunity to investigate the link between genetic and phenotypic variations as a consequence of hybridization after a secondary contact between divergent lineages that underwent range expansion. In particular, this research outlined different dispersal profiles associated with mitochondrial genotype, contributing to the growing number of studies unravelling the eco-evolutionary implications of mitochondrial variations within the POLS context. More broadly, this research furthers our understanding of the genetic correlation underlying phenotypic variation of dispersal syndromes. Indeed, our results suggest that a slow-thorough dispersal syndrome is a valid alternative strategy to the fast one that might be crucial for a successful dispersal process. Furthermore, we suggest the existence of slow-thorough dispersal mitotypes that could be drivers for mitochondrial introgression in this species of fire salamander, substantiating the adaptive introgressive hybridization hypothesis to explain such widespread genetic pattern within secondary contact zones. Overall, this study contributes to shedding light on the process of hybridization during range expansions and its eco-evolutionary implications, supporting evidence that geographic patterns of genetic variation can promote and be promoted by geographic variation in behaviour.

To conclude, our work opens new research prospects in the evolution of dispersal strategies in organisms displaying complex life cycles, such as amphibians, and raises new interesting perspectives about the evolutionary pathways that contribute to the diversification of phenotypic traits during dispersal process.

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8. AKNOWLEDGEMENTS

I would like to thank my tutor Prof. Daniele Canestrelli, who opened my mind for a different view of the natural world with the eyes of an eco-evolutionary biologist, giving me the opportunity to work on innovative and crucial issues of ecology. A special thanks to Prof. Claudio Carere for providing me the fundamental bases of ethology that allowed me to carry out the behavioural tests and process the data. Furthermore, I would like to thank Prof. Giuseppe Nascetti for giving me the opportunity to do this PhD at the University of Tuscia.

I also thank Roberta Bisconti ed Andrea Chiocchio for the help and the support in every step of this research, and another special thanks to the DEB Michela Paoletti and for contributing to laboratory analysis and CISMAR colleagues Giada Spadavecchia, Giacomo Grignani and Alessandro Carlini for the help in the field and the management of animals.

Finally, a huge thank to my family and Francesco for the fundamental support during all my academic career and for believing in me.