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Evolution and Persistence of Paedomorphosis in Newts (Amphibia, Urodela)

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Εξέλιξη και Διατήρηση της Παιδομόρφωσης σε Τρίτωνες (Αμφίβια, Ουρόδηλα)

Τόλη Ελισάβετ-Ασπασία

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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General Introduction

Κεφάλαιο 1

Γενική Εισαγωγή

1.1 Phenotypic plasticity: The example of paedomorphosis

Phenotypic plasticity is a strategic response in environmental heterogeneity, and it is common in many taxa, affecting different aspects of an organism such as morphology, physiology and even behavior (Miner et al. 2005). It grants organisms the flexibility to adapt in changing environments, leading to ecological success of populations. For instance, several plants exhibit alternative phenotypes in response to different environmental pressures such as the common dandelion (*Taraxacum officinale*) which shows different morphological features and seed dispersal in different light densities and qualities (Brock et al. 2005). A special case of phenotypic plasticity, where the environment is the major trigger leading to multiple distinct outcomes, is termed polyphenism (Yang & Andrew Pospisilik 2019). Such cases are the castes of honeybee (*Apis mellifera*), where each individual can become either a worker or a queen (Shuel & Dixon 1960), or the winged vs. wingless female aphids during the parthenogenesis process (Dixon 1998). The interaction between genotype and environment has been the center of much research regarding the expression and persistence of different phenotypes and the evolutionary responses in ever-changing habitats (Forsman 2014).

Plasticity is common in amphibian species affecting their morphology, growth rates, behavior, and metamorphosis. During their life cycle, amphibians go through metamorphosis from the aquatic larval stage to the terrestrial stage (Wilbur & Collins 1973). However, many salamander species exhibit a polyphenism called paedomorphosis, in which sexually matured individuals retain larval external morphology such as gill slits and external gills (Wilbur & Collins 1973, Whiteman 1994). There have been reports of paedomorphosis in 57 species of newts and salamanders and is considered an important evolutionary strategy (Semlitsch & Wilbur 1989, Duellman & Trueb 1994, Denoël et al. 2005). Paedomorphosis can be obligatory, like the well-known axolotl (*Ambystoma mexicanum*) that rarely reaches maturity without undergoing metamorphosis. Other salamander species exhibit facultative paedomorphosis, where they maintain the ability to metamorphose later in their lifespan (Duellman & Trueb 1994). Crossbreeding experiments and artificial selection of paedomorphosis suggest a genetic basis and the involvement of specific genes in the expression of this strategy (Semlitsch & Wilbur 1989, Voss 1995, Voss et al. 2003). A major quantitative trait locus linked with thyroid hormone responses, has been identified as the factor regulating the timing of metamorphosis and the expression of paedomorphosis in *A.*

mexicanum (Voss & Shaffer 1997, Voss & Smith 2005, Page et al. 2013). Nevertheless, the expression and maintenance of facultative paedomorphosis have been associated with several environmental factors (Denoël & Ficetola 2014).

There are two processes that produce paedomorphic phenotypes: a) neoteny, where the development is retarded relative to the sexual maturation and b) progenesis, where sexual maturity is accelerated relative to the rest of development (Gould 1977). Confounding results suggest that both ontogenetic trajectories coupled with local environmental conditions are responsible for the expression of paedomorphosis in different populations of the same species, making it a difficult task to decouple the underlying ecological processes (Whiteman 1994, Denoël & Joly 2000, Denoël et al. 2005).

1.2 Fitness trade-offs

Facultative paedomorphic species can enter the terrestrial habitat when the aquatic dwelling becomes hostile by undergoing metamorphosis, and this is an extreme example of how phenotypic plasticity benefits individuals in new environmental conditions (Whiteman 1994). This significant shift gives an advantage by enabling exploitation of different niche and resources, offering better chances of survival and persistence to environmental heterogeneity (Denoël 2006).

The "paedomorph advantage" hypothesis suggests that paedomorphic individuals have a fitness advantage over metamorphosing ones in the current environmental conditions. It was proposed by Wilbur and Collins (1973), and it describes the development and maintenance of paedomorphs in favorable aquatic conditions surrounded by harsh terrestrial habitat. Alternatively, when unfavorable aquatic conditions prevail (e.g. high population density, high predatory pressure, or low levels of food resources), the "best of a bad lot" hypothesis (Whiteman 1994) may predict the persistence of paedomorphosis. Here, paedomorphosis is maintained because it gives an advantage on an early age and smaller size rather than delaying reproduction to get a larger size, optimal for metamorphosis (Whiteman 1994). However, alternative mechanisms may explain the exhibition of paedomorphosis, since studies of paedomorphosis in newts show that life history traits, such as fecundity and mating success, can affect the decision to either metamorphose or become a paedomorph (Ryan & Semlitsch 1998).

In several populations, paedomorphic and metamorphic individuals coexist at the same breeding habitat, thus raising questions regarding the fitness advantages and the ecological factors that promote the expression of these alternative strategies (Whiteman 1994, 1997). By exhibiting both morphotypes, newts can decrease intraspecific competition by utilizing different trophic niches (Lejeune et al. 2018). At the same time, several newt populations, exhibit deviations from a 1:1 sex ratio for each morphotype (Whiteman 1994). Since there is sexual compatibility between paedomorphs and metamorphs (Oromi et al. 2016), sex-specific fitness pay-offs can influence this environmentally induced polymorphism.

1.3 Conservation importance

Notably in the last decades, amphibian populations have been decreasing due to climate change, habitat destruction and/or fragmentation, and the introduction of invasive aquatic species (Blaustein & Kiesecker 2002, Beebee & Griffiths 2005, Hartel et al. 2007, Denoël 2012, Denoël et al. 2019). Small aquatic bodies are important environments for freshwater biodiversity and suitable habitats for many amphibian species such as newts. However, such habitats are highly susceptible to threats like desiccation and pollution (Biggs et al. 2017).

Especially, paedomorphic newt populations are facing severe declines and extirpations, since they are highly depended on small aquatic bodies like ponds and ditches. Severe declines of paedomorphic populations have been observed after fish introductions (Denoël et al. 2005, Denoël et al. 2016, Toli et al. 2020), while pond desiccation increases metamorphosis rates leading to decreasing proportions of paedomorphs (Mathiron et al. 2017). Both anthropogenic pressures and the climate crisis are major threats leading to decline of the polyphenic trait of paedomorphosis, which in turn could lead to the loss of potentially adaptive plasticity and a lower ability to adapt to changing environments.

1.4 The Greek smooth newt, *Lissotriton graecus* (Wolterstorff, 1906)

Newts are small, tailed amphibians that belong in the family *Salamandridae* and are widely distributed in the Northern hemisphere. They are defined as those salamanders that have a clear aquatic stage as adults and in some cases, they can spend their whole life in water, in contrast to species which are terrestrial in the adult stage (Griffiths & Teunis 1996).

The Greek smooth newt, *Lissotriton graecus*, is a small-bodied newt that belongs in the *Lissotriton* species complex, which currently comprises of six species (*L. vulgaris*, *L. montandoni*, *L. kosswigi*, *L. graecus*, *L. lantzi* and *L. schmidtleri* (Wielstra et al. 2018) and is found in Greece along with *L. schmidtleri* (Wielstra et al. 2018). It is distributed in the southwest Balkan Peninsula, while in Greece it occurs in the mainland, Peloponnese and the Ionian islands (Figure 1.1).

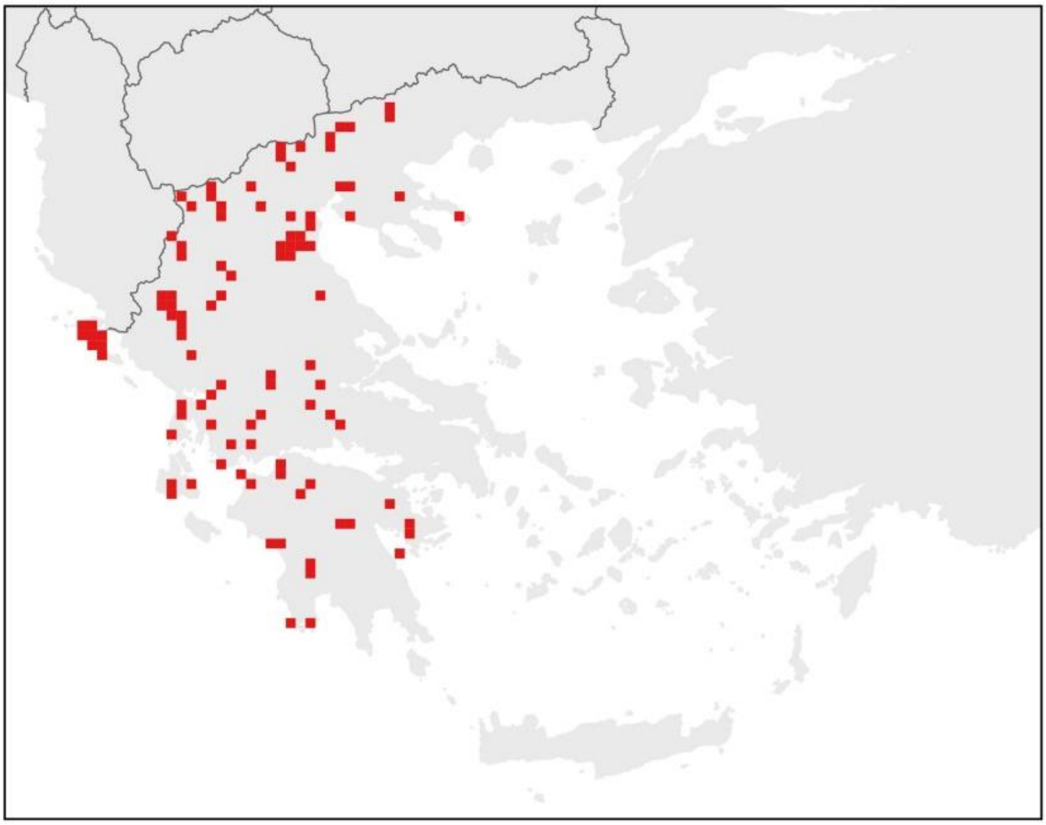

Figure 1.1: Distribution of *L. graecus* in Greece (Atlas of Amphibians and Reptiles in Greece; http://herpatlas.gr/herp-finder/lissotriton-graecus/).

Εικόνα 1.1: Χάρτης επιβεβαιωμένων τοποθεσιών του είδους *L. graecus* στην Ελλάδα (Άτλας Αμφιβίων και Ερπετών της Ελλάδας, http://herpatlas.gr/herp-finder/lissotriton-graecus/).

The species occurs in a wide range of habitats, from natural and artificial small ponds to larger lakes, while the terrestrial landscape includes deciduous/mixed woods, grassland, and cultivated areas. Connectivity between the water bodies, macrophyte cover and absence of fish are important factors for predicting its occurrence (Bounas et al. 2020). Usually, it lives in sympatry with other newt species like *Ichthyosaura alpestris* and *Triturus macedonicus*. Greek smooth newts are carnivorous, and their diet consists of invertebrates such as crustaceans, insect larvae, worms, and slugs. They also have cannibalistic tendencies in overcrowded conditions and prey upon eggs and larva or even smaller newts (Valakos et al. 2008, Pafilis et al. 2020).

Facultative paedomorphosis has been reported in many European newts, especially in members of *Lissotriton* (Kalezić & Džukić 1985, Litvinchuk et al. 1996, Çevik et al. 1997, Litvinchuk 2001, Sidorovska et al. 2003, Sotiropoulos et al. 2008, Denoël et al. 2009, Skorinov et al. 2009, Çiçek & Ayaz 2011, Covaciu-Marcov et al. 2013, Gvoždík et al. 2013, Picolli 2013, Bozkurt et al. 2015, Bozkurt et al. 2016, Sotiropoulos et al. 2017, Denoël et al. 2019, Sotiropoulos et al. 2020). In Greece, facultative paedomorphosis has been reported for four species of newts; the alpine newt, *Ichthyosaura alpestris* (14 localities; Denoël et al. 2001, Denoël 2004, Sotiropoulos et al. 2021), the Greek smooth newt, *Lissotriton graecus* (six localities; Sotiropoulos et al. 2008, Sotiropoulos et al. 2017, Sotiropoulos et al. 2021), the Anatolian smooth newt, *Lissotriton schmidtleri* (one locality; Sotiropoulos et al. 2020) and the Macedonian crested newt, *Triturus macedonicus* (seven localities; Sotiropoulos et al. 2017, Sotiropoulos et al. 2021).

The species is under protection (National Legislation 67/1981 and Bern Convention Appendix III) and is listed as Least Concern (LC) in the IUCN Red list (IUCN, 2021). However, several studies show the decreasing of newt populations due to climate change and habitat fragmentation (Stuart et al. 2008, Denoël 2012). Due to their requirements for higher water quality, newts are more vulnerable to habitat degradation, drought, and fish introductions, especially paedomorphic populations since they spend most (or entire) of their life in aquatic habitats (Denoël et al. 2005, Denoël 2012, Mathiron et al. 2017, Toli et al. 2020).

1.5 The Study Population and its Habitat

In this study, I focused on a Greek smooth newt population, located in an artificial fishless pond in the University of Ioannina Campus, Greece (39°36'N 20°50'E, 497m a.s.l). The pond is circular with a mean diameter of 17m and maximum depth of 1.5m (Figure 1.2). Early in the spring, 75% of the pond is covered by submerged aquatic vegetation. A range of invertebrate species have been sampled in the pond, including Copepoda, Cladocera, Diptera, Hemiptera, Anisoptera, Trichoptera, Coleoptera and Mollusca. The surrounding habitat is characterized mostly by abandoned cultivations and extended pastures of herbaceous vegetation, with sparse deciduous trees (Figure 1.2). The pond has not been used for livestock or cultivation watering for the last 15 years and is used as a breeding site for several amphibian species, such as *Bufo bufo*, *Hyla arborea*, *Pelophylax epeiroticus*, *Bufo viridis* and *Triturus macedonicus*. Cases of paedomorphosis have been recorded for the Macedonian Crested newt as well, highlighting the importance of the study site (Sotiropoulos et al. 2017).

Figure 1.2: The study site located in University of Ioannina Campus (photo by E. Toli).
Εικόνα 1.2: Πληθυσμός μελέτης στην Πανεπιστημιούπολη Ιωαννίνων (Φωτ. από Ε. Τόλη).

The study population has been the focus of several research projects since 2015. According to mitochondrial DNA analysis, the population belongs to the ‘Clade C’, a well-defined ancestral lineage that coincides with a refugial population (Pabijan et al. 2015, Wielstra et al. 2018). The studied population shows high levels of facultative paedomorphosis (85.2-100%) (Sotiropoulos et al. 2017), while monthly counts using dip-nets showed considerable deviation from a 1:1 sex ratio and contrasting sex ratios within each morph (Figure 1.3), implying fitness differences specific to the two sexes within each morph.

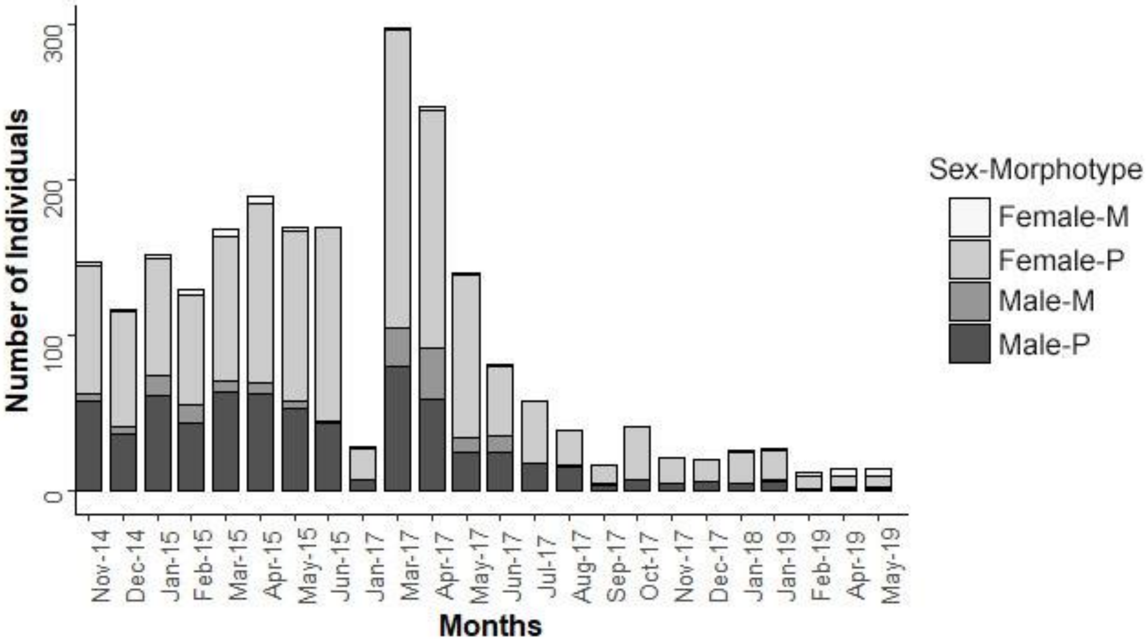

Figure 1.3: Number of newts caught in each sampling session according to sex and morphotype (M: metamorphic, P: paedomorphic) (Toli et al. 2017).

Εικόνα 1.3: Αριθμός ατόμων που βρέθηκαν σε κάθε δειγματοληψία ανάλογα με το φύλο και τη μορφή (M: μεταμορφωμένα, P: παιδομορφικά) (Toli et al. 2017).

1.6 Aims

The aim of this dissertation was to explore possible factors and their relative effects on the expression and persistence of facultative paedomorphosis in a natural population of the Greek smooth newt. Specifically, the research questions concern the study of the evolutionary processes involved in the occurrence of facultative paedomorphosis and the persistence of the paedomorphic population on the long term, and under different environmental stressors.

Behavioral and ecological experiments were performed to study the fitness of the alternative phenotypes. Specifically, reproductive behaviors such as male display, egg laying and larval survival were measured since they constitute important components of individual reproductive fitness. Additionally, by using genetic tools like Single Nucleotide Polymorphisms (SNPs), I aimed to evaluate the genetic structure between the alternative morphs of the studied population. Genetic markers can identify the presence of gene flow and sexual compatibility between the two morphotypes, along with genes potentially under selection linked to each phenotype. Moreover, I evaluated the levels of individual heterozygosity and possible correlations with the reproductive fitness components within each sex and morph, and unraveled possible genetic loci and polymorphisms associated with quantitative phenotypic traits.

Combining both fitness and genetic data in Population Viability models, I provide an estimate of the levels of gene diversity and viability of the population on the long-term, along with the likelihood of extinction risks and persistence of the paedomorphic phenotype under different environmental conditions.

In addition, an effort was made to clarify the ecological interactions of paedomorphic newts with invasive fish species. Alien fish introductions are well established around the globe and are a matter of concern in the study region as well. Through laboratory experiments, I assessed possible disturbances in the behavior of paedomorphic individuals in the presence of fish, as well as whether the presence of fish promotes or accelerates the rate of metamorphosis.

The results of this dissertation are expected to contribute significantly to the understanding of the underlying processes on the expression and persistence of facultative paedomorphosis in newts.

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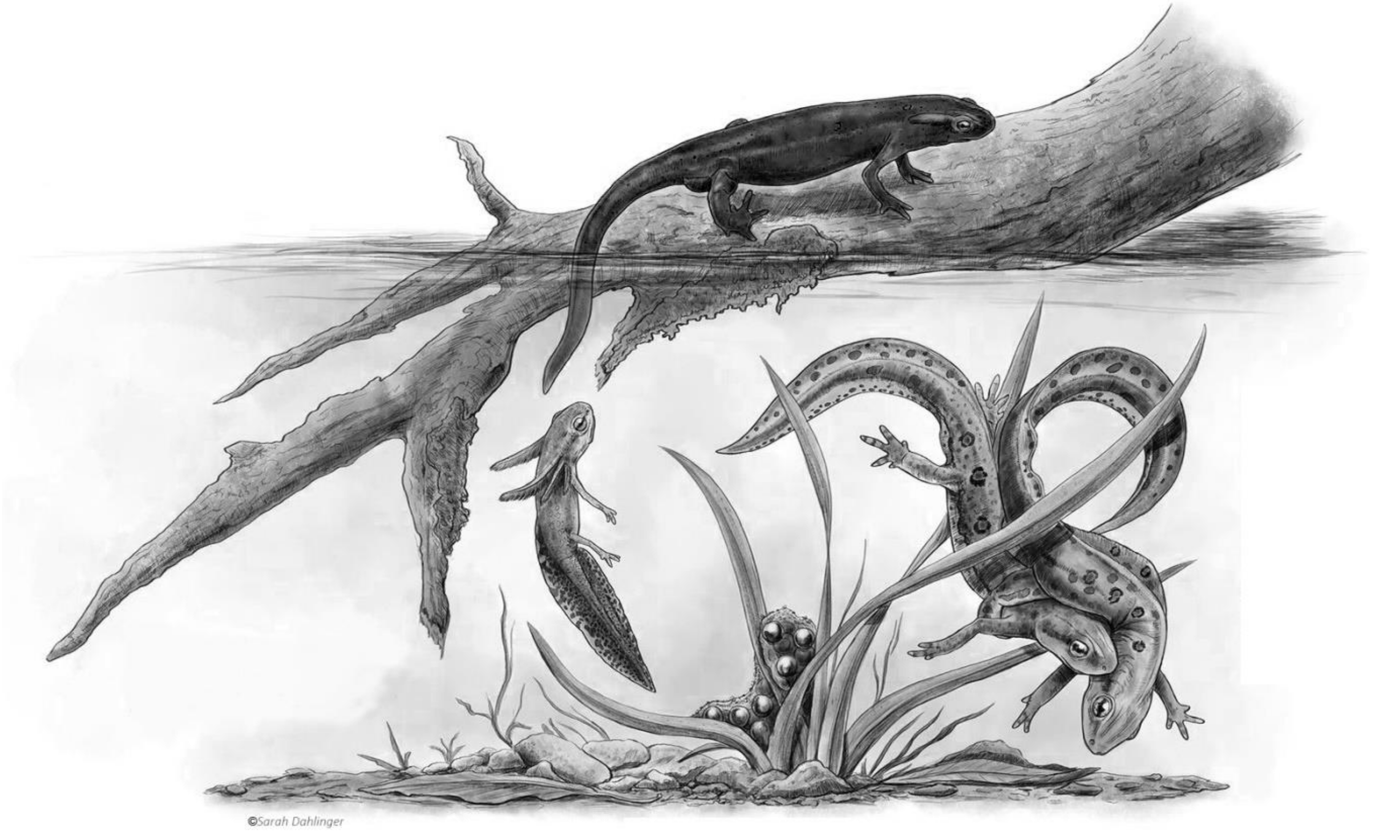
Chapter 2

What are the fitness differences between alternative morphs and sexes, regarding body condition and reproductive components?

Κεφάλαιο 2

Ποιες οι διαφορές στην αρμοστικότητα μεταξύ των εναλλακτικών μορφών και των φύλων ως προς τη σωματική κατάσταση και τις αναπαραγωγικές συνιστώσες;



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2.1 Introduction

Fitness is a key concept in evolutionary biology where each given genotype or phenotype contributes differently to the gene pool of the next generation, and without differences in fitness, natural selection and adaptation could not occur (Orr 2009). Depending on the prevailing environmental conditions, one morphotype might have greater fitness advantage than the other, contributing to the manifestation of polyphenisms in a population, such as paedomorphosis (Whiteman 1997). Fitness-related traits such as body condition, longevity, mating success and fecundity, are all correlated and contribute to the lifetime reproductive success of an individual (Coulson et al. 2006).

Body condition indices have been widely used as fitness proxies, indicating the underlying condition and health of an individual (Jakob et al. 1996). Higher body condition is linked with improved ability to cope with environmental changes, which may affect the lifetime reproductive success (Jakob et al. 1996, Jarvis 2015). In salamanders and newts, body size is correlated with higher fecundity and the expression of secondary sexual characteristics (Halliday & Verrell 1986, Andersson 1994). However, body condition alone may not be an adequate measure of fitness, since body condition changes seasonally, and reflects the current condition of an individual (Jarvis 2015, Wilder et al. 2016). Since newts exhibit complex and elaborate behaviors during the breeding season (Halliday 1990), incorporating fecundity and courtship behaviors is crucial in studies regarding reproductive fitness.

In *Lissotriton vulgaris*, metamorphic males enter the pond and prepare for the breeding season by exhibiting colorful patterns, dorsal crest, swallowed reproductive organs and a filament at the end of their tail, which increase the effectiveness of fanning (Halliday 1990). Simultaneously, paedomorphic males are already in the pond since they spend all their time in the water. Through their reproductive organs, both males and females secrete pheromones to attract the opposite sex (Arnold 1977, Arnold & Houck 1982, Belvedere et al. 1988, Kikuyama & Toyoda 1999, Treer et al. 2013).

Courtship behavior involves different stages where the male tries to attract female's attention through visual and chemical cues. The reproductive sequence in *Lissotriton sp.* is characterized by a stimulus-response chain, since the male in order to continue with each courtship step needs positive feedback from the female (Halliday 1990). The male begins by sniffing female's cloaca, gets in a position in front of her and employs tail and body movements, a phase known as display. If the female is interested, she may stay still and then start following the male. If the interest to the potential mate is maintained, the male deposits a spermatophore and through elaborate orientation behavior leads the female to pick up the spermatophore (Halliday 1975).

Male newts can produce several spermatophores over a short period of time, while female newts can pick up several spermatophores from different mates during one breeding season (Baker 1990). This strategy, of multiple inseminations, has been observed in salamander and newt populations, and seems to increase reproductive success (Osikowski & Rafiński 2001, Adams et al. 2005). Female choice is based in genetic dissimilarity and favorable secondary characteristics, such as body size, filament length and increased spermatophore deposition rate (Haerty et al. 2007, Hoeck & Garner 2007, Jehle et al. 2007). Interestingly, intermorph breeding is known to occur (Oromi et al. 2016), despite the clear morphological differences between paedomorphic and metamorphic males. Although, sexual secondary characteristics differ between the morphotypes, this seems to not affect mating success and intermorph encounters as studies show in the alpine newt (Denoël et al. 2001). Examples of assortative mating between the two morphotypes have been observed in a population of *Ambystoma talpoideum,* associated with earlier breeding of paedomorphs (Krenz & Sever 1995).

Female newts fertilize their eggs and deposit them singly in aquatic vegetation (Griffiths & Teunis 1996). They wrap the eggs on the leaves of water plants for protection, using their hind limbs, and abandon their eggs (Griffiths & Teunis 1996). After a few weeks, the first eggs are hatched, and larval development starts (Figure 2.1).

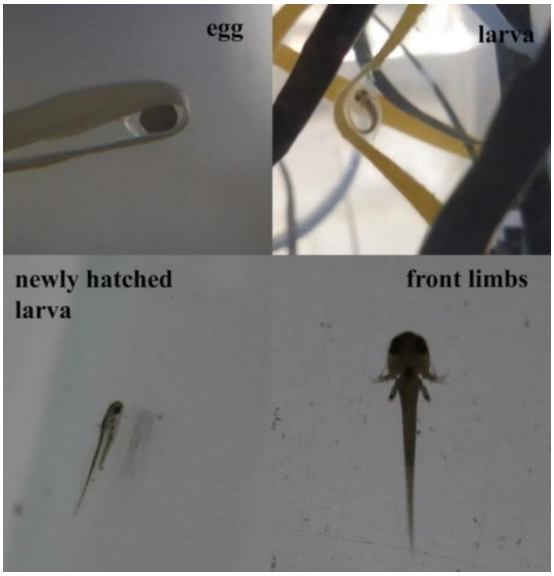

Figure 2.1: Representation of the development from an egg to the stage where larva displays forelimbs (photos by E. Toli).

Εικόνα 2.1: Τα αναπτυξιακά στάδια από το αυγό έως το στάδιο όπου η προνύμφη του τρίτωνα εμφανίζει μπροστινά άκρα. (Εικόνες από Ε. Τόλη).

In several weeks, larvae develop their forelimbs followed by the hind and they consume zooplankton such as water fleas and copepods (Bell & Lawton 1975). In several weeks, depending on the aquatic and terrestrial environmental conditions, larvae either metamorphose to terrestrial newts by resorbing their gills or become paedomorphic by acquiring sexual maturity while retaining their juvenile features such as gills and gill slits (Whiteman 1994).

The study population deviates from a 1:1 sex ratio and shows contrasting sex ratios within each morph (see General Introduction). Since sex ratio plays a significant role in mate choice and competition, affecting population growth rate and the viability of populations (Dyson & Hurst 2004), the observed ratios could imply fitness differences specific to the two sexes within each morph in the study population. Paedomorphosis is favored in permanent, predator-free waters, and individuals may choose this ontogenetic trajectory due to long term fitness advantage (Denoël et al. 2002). By remaining in the aquatic habitat, individuals may have greater access in energetic resources, hence enhance their reproductive success.

This chapter explores the proximate mechanisms of facultative paedomorphosis by using body condition and reproductive components as fitness proxies. More specifically, the reproductive components of fitness in a *Lissotriton graecus* population, exhibiting both morphs, were investigated through a two-phase experiment. In the first phase, courtship behaviors and mating preferences were observed. Females were placed with both paedomorphic and metamorphic males to examine potential differences in courtship behavior and sexual isolation between the alternative phenotypes. In the second phase, egg production, larval development and viability were recorded, in order to explore potential fitness differences between the two phenotypes.

2.2 Materials & Methods

2.2.1 Experimental Design

A total of 12 male and 61 female newts were caught using dip-nets from an artificial pond in Ioannina basin, Greece (39°36'N 20°50'E, 497m a.s.l) in January 2019 before the start of the breeding season. Their sexually maturity was verified based on a swollen cloaca (Denoël 2017). All individuals were brought directly to the laboratory in large containers filled with pond water and were placed randomly in 20L aquatic tanks (38x24 cm, 22 cm water depth).

Body Condition Index

Newts were anesthetized in a solution of MS-222 (0.3g/L) according to regulations and snout vent length (SVL, mm) and body weight (gr) were measured to the nearest 0.01mm and 0.01gr using a digital caliper and a scale. Body weight was regressed on body size after log transformation, and the residual distances from the linear regression line were used as proximate measures of individual fitness (Jakob et al. 1996).

Reproductive Components

After their recovery, newts were placed in 20L aquatic tanks (38x24 cm, 22 cm water depth) filled with dechlorinated tap water; temperature was maintained at an average 17°C and natural photoperiod was used (13h light-11h dark). All newts were fed *ad libitum* with black mosquito larvae (*Culex sp.*) and blood worms (*Chironomus sp.*). The water was renewed every five days using dechlorinated tap water from tanks stored at the experimental temperature. After the end of the experiments all newts were released at the breeding site.

1st experimental phase: Courtship behavior

For the courtship behavior experiment, 12 males (six metamorphic and six paedomorphic) and 19 females (6 metamorphic and 13 paedomorphic) were used. In each tank one metamorphic and one paedomorphic male were placed. One random female was placed to pair with either the paedomorphic or metamorphic male and each tank was then video recorded for 40 minutes (video recorders TurboX Act-150 were set at a resolution of 1280*720 pixels and 30 frames per second). After the video recordings, we removed the female and placed the next one. Encounters were at least one day apart, and the water was renewed every time to reduce the pheromones from the previous encounter.

From the videos we quantified the event of display (display *vs* no display: male employing tail fanning in front of the female), the duration of display, the male display latency (time in seconds from the entrance of the female to the first act of male display) and female responsiveness (responsive vs not responsive; female moving towards male) using the BORIS software for behavioral analyses (Friard & Gamba 2016).

2nd experiment: Egg production and larval development

A total of 48 paedomorphic and 13 metamorphic female Greek smooth newts were placed alone in 20L aquatic tanks (38x24 cm, 22 cm water depth) in the laboratory. Each female was provided with oviposition material, i.e., strips of plastic fixed on the bottom of the tank, simulating aquatic plants. Visual contact of neighboring females was blocked using thick paper between aquaria. The oviposition material was replaced every day and the number of deposited eggs of each female were counted and then transferred in new aquaria. Eggs that were not developing were removed daily. The act of egg laying, the total number of deposited eggs and the number of successfully hatched eggs were recorded daily. The experiment lasted for 24 weeks until all females stopped laying eggs and all surviving larvae had metamorphosed. The newly hatched larvae were transferred in aquaria and were fed with a stock of zooplankton. Larvae were measured with a digital caliper to the nearest 0.01mm and categorized in three developmental stages (Glucksohn 1931). In stage I larvae were characterized by their developed gills, in stage II larvae developed their forelimbs, while in stage III, larvae had reached the sub-adult stage (metamorphosis) and were returned to the breeding site.

2.2.2 Statistical Analyses

Body Condition Index

Regression analyses and Welch's t-test were performed to test for differences in body condition between paedomorphic and metamorphic newts within each sex in RStudio v. 3.4.1. (Team R. 2015). Welch's t-test was used due to unequal sample variances.

Reproductive Components

1st experiment: Courtship behavior

Courtship data were analyzed using Generalized Linear Mixed Models (GLMM) and Linear Mixed Models (LMM) as implemented in package “lme4” (Bates et al. 2014) in R Studio v. 3.4.1. (Team R. 2015). I fitted a GLMM on binomial data of display (display vs no display), using log(SVL), male morph (paedomorph vs metamorph), female morph (paedomorph vs metamorph) and their interaction as fixed effects, and male and female identity as random effects. LMM models were fitted for duration and latency, using log(SVL), male morph, female morph and their interaction as fixed effects and male and female identity as random effects. Models were tested for overdispersion and normality. Pairwise comparisons were analyzed post hoc using the “lsmeans” package (Lenth 2016). Female responsiveness was fitted in a GLMM with binomial distribution, using latency, duration and male morph as fixed effects and male and female identity as random effects.

2nd experiment: Egg production and larval development

In order to investigate probable associations between egg laying (female that laid eggs vs female that didn’t) with log(SVL), a generalized model with binomial distribution was used, setting the morph as a fixed effect. Generalized models were used to analyze the relationships between fitness proxies and body size in R (Team R. 2015). Different models for number of laid eggs, the number of successfully hatched eggs, and the number of surviving larvae were fitted using log(SVL) and morph as fixed effects. Since these traits are count data, models were tested for overdispersion, using a negative-binomial error distribution implemented in the R package MASS (Venables & Ripley 2002). Larval survival was reported for each of the three developmental stages. Differences within each stage between the morphs (larva from either a paedomorphic or a metamorphic female) were tested using chi square analysis in RStudio v. 3.4.1. (Team R. 2015).

2.3 Results

Body Condition Index

The slopes of regression analysis of body weight on body size, did not differ in males but differed significantly in females (Table 2.1), in which the regression slope was higher in paedomorphs than in metamorphs (Figure 2.2). Body condition scores were statistically different in females, with paedomorphic females exhibiting higher body condition than metamorphic ones (Table 2.1). However, body condition scores in males were not statistically different between the two morphotypes (Table 2.1).

Table 2.1: Comparisons of the residuals from the linear regression of logWeight on logSVL between the two morphs in the Greek smooth newt.

Πίνακας 2.1: Συγκρίσεις των υπολειμματικών τιμών των γραμμικών παλινδρομήσεων (logWeight /logSVL) μεταξύ των δύο μορφών του ελληνικού κοινού τρίτωνα για κάθε φύλο.

Sex		**Paedomorphs**		**Metamorphs**					
	N	**Mean residual**	**S.E.**	**N**	**Mean residual**	**S.E.**	**Welch's t-test**	**d.f.**	**P-value**
Females	48	0.162	0.015	13	0.032	0.016	-5.781	33.408	**<0.001**
Males	6	0.117	0.016	6	0.074	0.026	-1.389	8.558	0.199

N: number of individuals, S.E.:Standard Error, d.f.: degrees of freedom

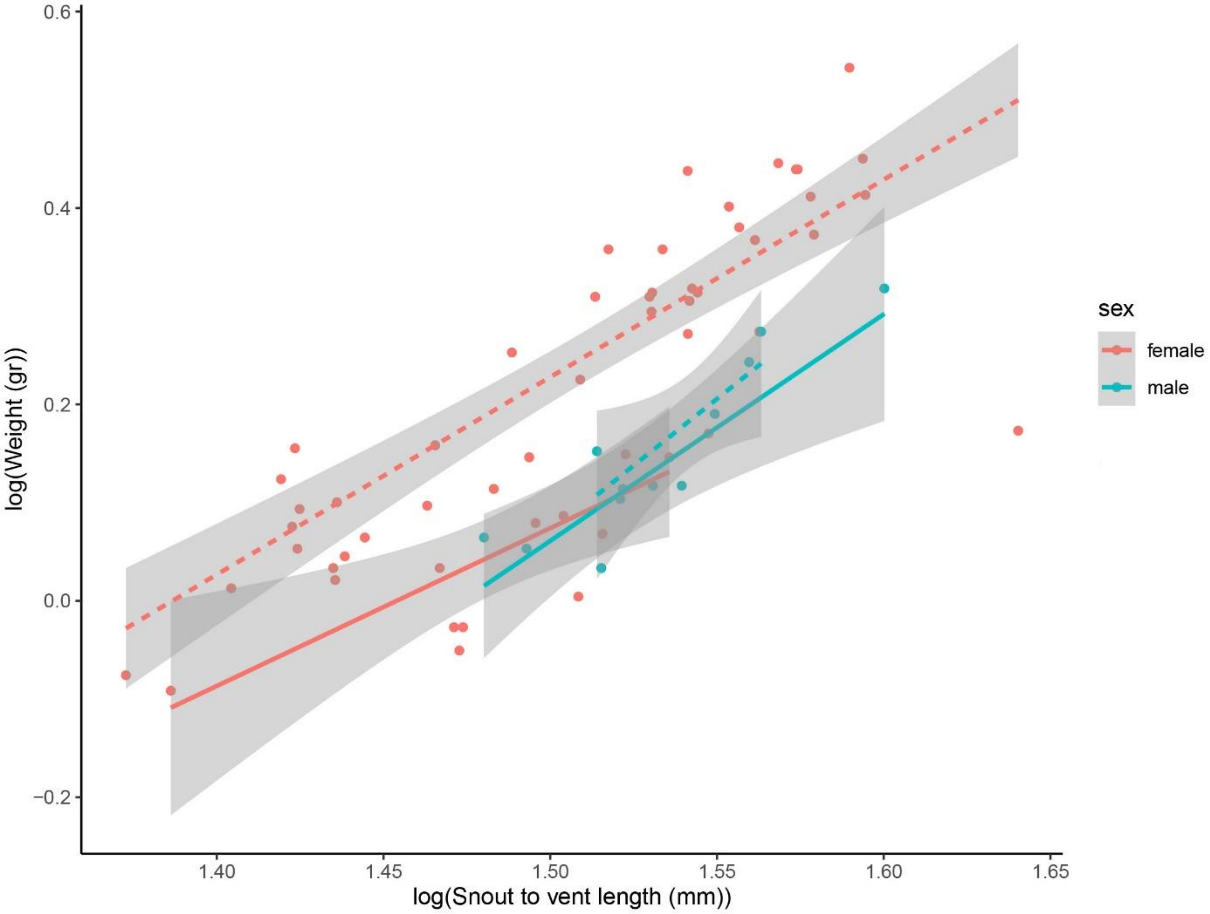

Figure 2.2: Linear regressions of logWeight on logSVL of metamorphs (solid line) and paedomorphs (dashed line) in female and male newts.

Εικόνα 2.2: Γραμμικές παλινδρομήσεις (logWeight/logSVL) για τους μεταμορφωμένους (κανονική γραμμή) και παιδομορφικούς (διακεκομμένη γραμμή) τρίτωνες για κάθε φύλλο.

Reproductive Components

1st experiment: Courtship behavior

A significant effect of male and female morph interaction (GLMM: χ^2=6.125, p=0.013) was found on male display. Post hoc results are summarized in Table 2.2 for the significant interaction. When female paedomorphs were present, seven male metamorphs and nine male paedomorphs engaged in courtship behavior, while when female metamorphs were present four male metamorphs and one male paedomorph started displaying (Figure 2.3). No statistically significant effect of log(SVL) (GLMM: χ^2=0.001, p=0.965), male morph (GLMM: χ^2=0.022, p=0.880), female morph (GLMM: χ^2=1.718, p=0.189) and their interaction (GLMM: χ^2=1.636, p=0.201) was found on duration. No statistically significant effect of log(SVL) (GLMM: χ^2= 0.171, p= 0.678), male morph (GLMM: χ^2= 1.148, p=0 0.284), female morph (GLMM: χ^2=0.033, p=0.854) and their interaction (GLMM: χ^2= 1.562, p= 0.211) was found on latency time.

Female responsiveness was found to be affected by the duration of display (GLMM: χ^2=4.518, p=0.033, Figure 2.4).

Table 2.2: Pairwise comparisons obtained by “lsmeans” package (Tukey’s method) of the effect of female and male morph interaction on display. Statistically significant values are shown in bold.

Πίνακας 2.2: Συγκρίσεις κατά ζεύγη με τη μέθοδο Tukey για την επίδραση της μορφής των θηλυκών και αρσενικών ατόμων κατά την ερωτοτροπία. Οι στατιστικά σημαντικές τιμές φαίνονται σε έντονη γραφή.

Encounters		**Estimate**	**SE**	**df**	**Z ratio**	**P value**
Metamorphs Females-Metamorphs Males	Paedomorphs Females-Metamorphs Males	-0.607	0.902	NA	-0.673	0.907
Metamorphs Females-Metamorphs Males	Metamorphs Females-Paedomorphs Males	2.284	1.513	NA	1.510	0.431
Metamorphs Females-Metamorphs Males	Paedomorphs Females-Paedomorphs Males	-1.969	1.158	NA	-1.701	0.323
Paedomorphs Females-Metamorphs Males	Metamorphs Females-Paedomorphs Males	2.891	1.578	NA	1.832	0.258
Peadomorphs Females-Metamorphs Males	Peadomorphs Females-Paedomorphs Male	-1.362	0.880	NA	-1.548	0.408
Metamorphs Females-Paedomorphs Males	Paedomorphs Females-Paedomorphs Males	-4.253	1.457	NA	-2.919	**0.018**

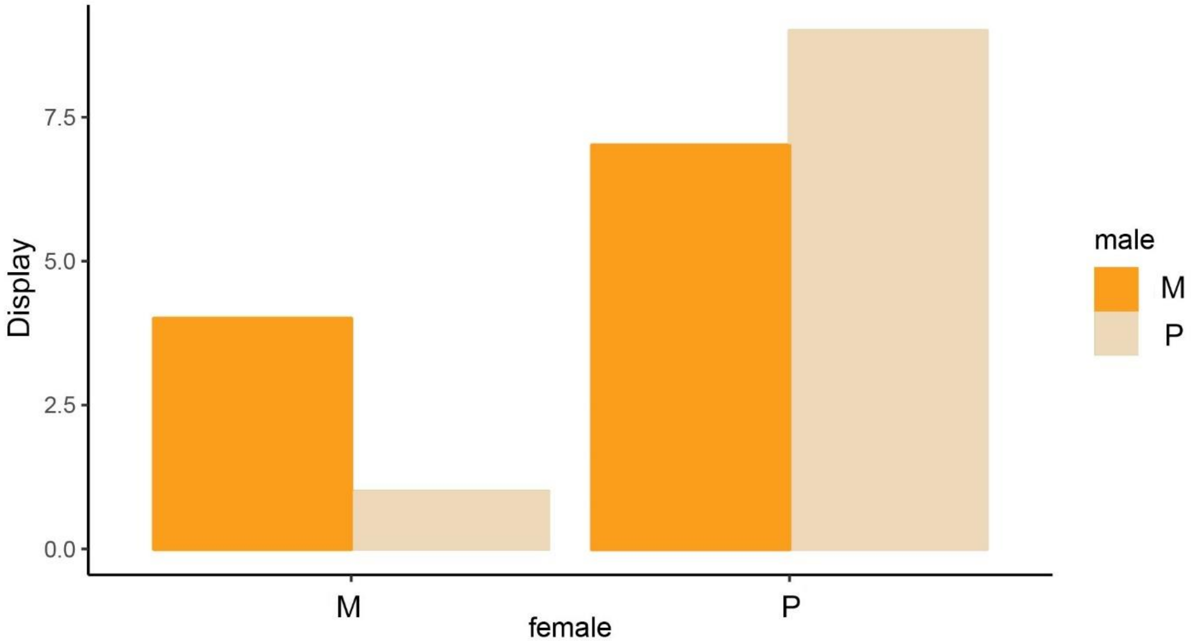

Figure 2.3: Paedomorphic (P) and metamorphic (M) male newts that engaged in display (y axis represent the number of male individuals) in the presence of each female morph (M: metamorphic, P: paedomorphic).

Εικόνα 2.3: Παιδομορφικοί και μεταμορφωμένοι αρσενικοί τρίτωνες που συμμετείχαν σε ερωτοτροπία υπό την παρουσία θηλυκών τριτώνων της κάθε μορφής.

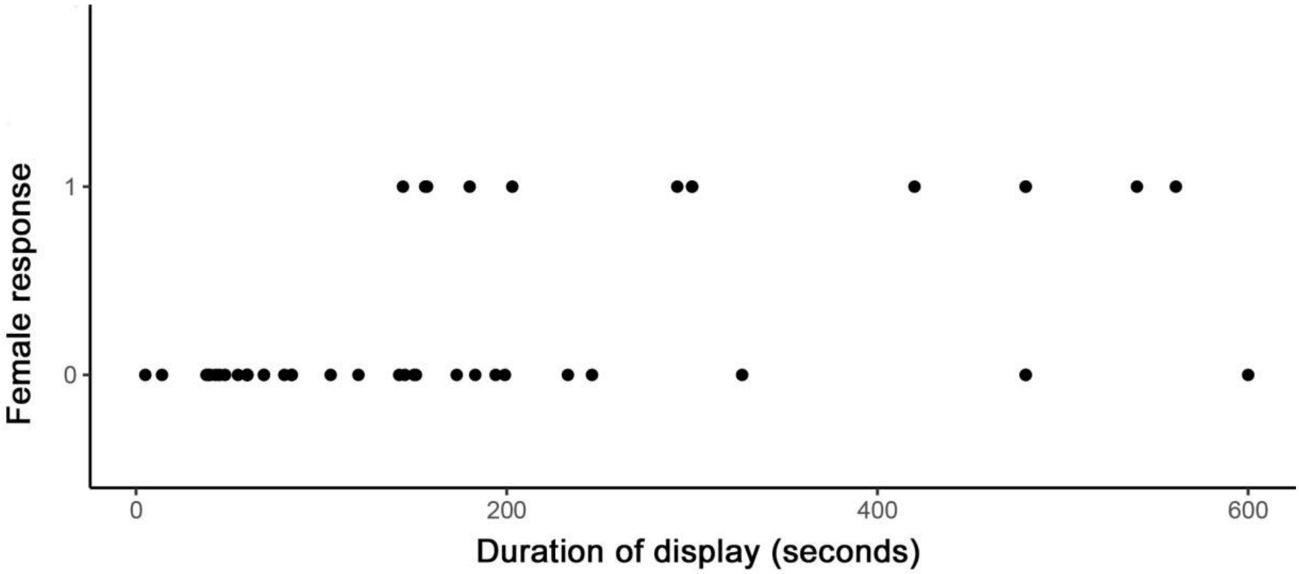

Figure 2.4: Response of female newts (positive 1 vs negative 0) according to the duration of male display.

Εικόνα 2.4: Ανταπόκριση των θηλυκών τριτώνων (θετική 1 vs αρνητική 0) σύμφωνα με τη διάρκεια ερωτοτροπίας των αρσενικών τριτώνων.

2nd experiment: Egg production and larval development

A total of 12 metamorphic and 36 paedomorphic female newts deposited 3,785 eggs in total, of which 60.3% hatched during the experiment. Egg laying (females that laid eggs vs females that did not) was found to be affected by log(SVL) (χ^2= 3.909, p= 0.048), but not by female morph (χ^2= 3.250, p= 0.071). Morph had a significant effect on number of eggs (GLM: χ^2= 5.369, p= 0.020) and number of successfully hatched eggs (GLM: χ^2= 3.724, p= 0.053). Metamorphic females deposited more eggs than paedomorphic ones (Mean number of eggs± Standard Error: metamorphic 114±16 and paedomorphic 67±12, Figure 2.5), of which more eggs from metamorphic females were successfully hatched (Mean number of hatched eggs± Standard Error: metamorphic 75±10 and paedomorphic 38±8, Figure 2.5). Log(SVL) had a significant effect on the number of eggs (GLM: χ^2= 5.309, p=0.021) and the number of survived larvae till the sub-adult stage (GLM: χ^2= 15.156, p<0.001), but not on the number of hatched eggs (GLM: χ^2= 1.977, p= 0.159).

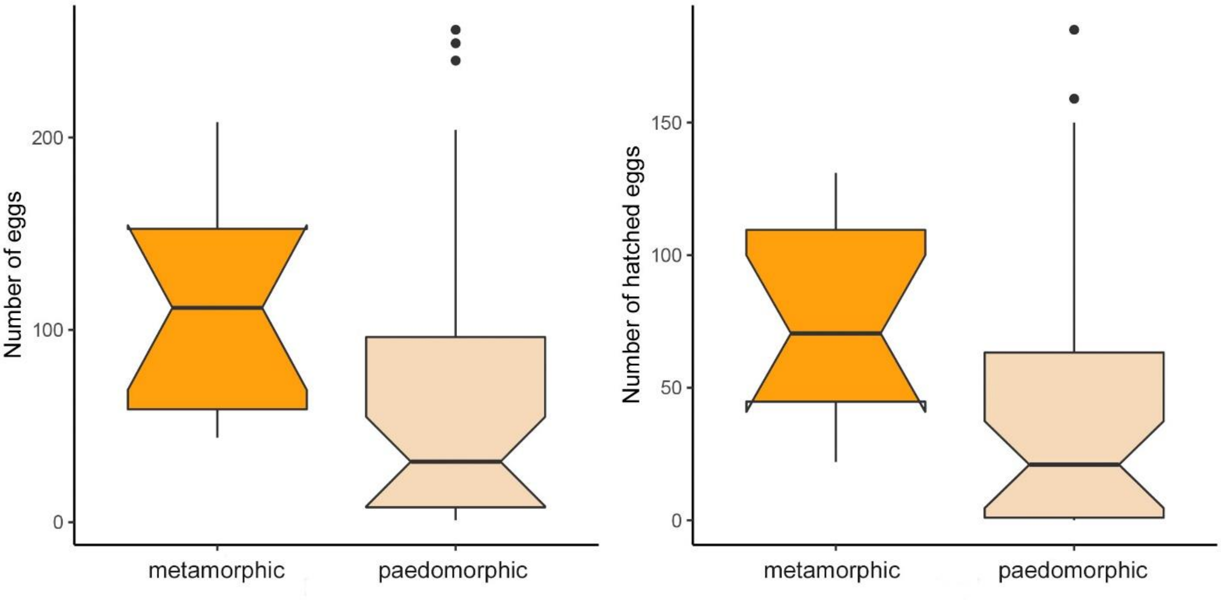

Figure 2.5: Number of eggs and number of successfully hatched eggs of female Greek smooth newts according to each morph (paedomorphic vs metamorphic). Whiskers represent 95% of Confidence Intervals and notches represent the median values.

Εικόνα 2.5: Αριθμός αυγών και αριθμός εκκολαπτόμενων αυγών των θηλυκών τριτώνων του ελληνικού κοινού τρίτωνα για κάθε μορφή (παιδομορφικά και μεταμορφωμένα). Παρουσιάζονται τα 95% διαστήματα εμπιστοσύνης και οι εγκοπές παρουσιάζουν τις μέσες τιμές.

Larval survival from hatching to the sub-adult stage (metamorphosis) was estimated to 13.25%. Results of larval survival in each developmental stage are summarized in Table 2.3. There is a significant association between larval survival and morph of the female parent ($\chi^2 = 14.569$, d.f. = 2, $p < 0{,}001$ Figure 2.6).

Table 2.3: Life table and larval survival (%) in each developmental stage from metamorphic and paedomorphic female newts. n: number of individuals.

Πίνακας 2.3: Ποσοστά επιβίωσης για κάθε στάδιο για μεταμορφωμένα και παιδομορφικά θηλυκά άτομα. n: αριθμός ατόμων.

Female parent	**Number of female genitors**	**eggs**	**hatched**	**Stage I**		**Stage II**		**Stage III**	
				n	**%**	**n**	**%**	**n**	**%**
Metamorphic	12	1,369	904	608	67.30	447	73.50	25	5.60
Paedomorphic	36	2,316	1,377	1,060	76.90	776	73.20	196	25.20
Total	48	3,785	2,281	1,668	73.10	1,223	73.30	221	18.10

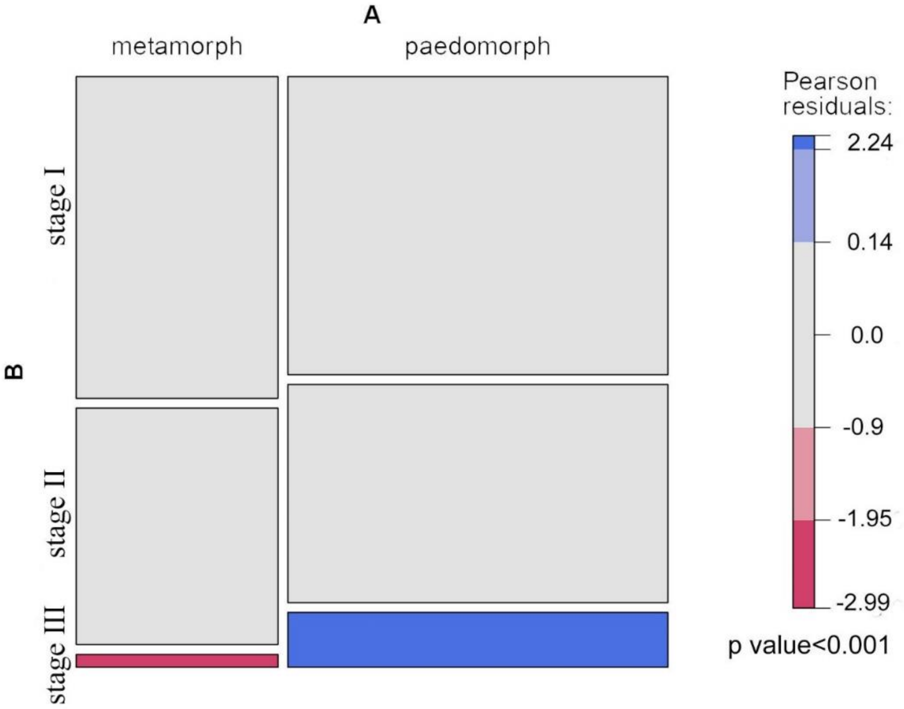

Figure 2.6: Contribution to the total Chi-square score of each category. Positive values (blue) in cells specify a positive association while negative values (red) imply a negative association between the corresponding row and column variables. The size of squares is proportional to the amount of the cell's contribution.

Εικόνα 2.6: Ο έλεγχος χ^2 δείχνει σημαντική συσχέτιση της βιωσιμότητας των προνυμφών με τη μορφή του θηλυκού ατόμου. Το στάδιο III συσχετίζεται θετικά με τη παιδομορφική μορφή και αρνητικά με τη μεταμορφωμένη.

2.4 Discussion

The data presented here suggest that multiple evolutionary mechanisms can promote facultative paedomorphosis in the studied Greek smooth newt population and reveal sex-specific differences in fitness.

Here, facultative paedomorphosis may maintained by fitness benefits in paedomorphic females. According to the body condition index, paedomorphic females seem to exhibit better body condition than metamorphs, supporting the "paedomorphosis advantage hypothesis". However, no indications of fitness differences between the morphs were found in male newts. Body size is highly correlated with lipid storage and energy, which are crucial in fecundity and reproduction (Denoël et al. 2005, Jaworski et al. 2018), hence body size may be more significant in females than males. Switching habitats and becoming metamorphic is time and energy consuming, and individuals might avoid the cost and gain long-term advantages by remaining paedomorphic (Denoël et al. 2002).

My experiments showed that body size has a significant positive effect in fecundity. In both morphotypes, larger females laid more eggs and had higher numbers of larvae that reached the sub-adult stage. Although, paedomorphic females laid fewer eggs, of which fewer hatched into newly larvae than metamorphic ones, they produced more larvae that reached metamorphosis and had a better outcome in reproduction. This confirms the hypothesis that higher body size is positively associated with fecundity, and in our study population, paedomorphic females have higher body size, hence, leaving more viable offspring.

Notably, I did not observe any fitness differences in body condition and courtship behavior between male paedomorphs and male metamorphs. The sex ratio of the study population is male biased towards metamorphic newts, which supports the "male escape hypothesis". Male newts metamorphose earlier than females in drying and/or stressful conditions (Mathiron et al. 2017, Toli et al. 2020), and may explain the female biased ratios of paedomorphs in the wild (Whiteman 1997). However, the study pond is isolated and permanent, without fish making it a suitable habitat for paedomorphic newts (Denoël & Ficetola 2014, Bounas et al. 2020). While, for male paedomorphs, it may be more favorable to stay in the water in order to get earlier access to female newts in the next breeding period, metamorphosis could be a way to lessen intraspecific competition between males by utilizing different trophic niches (Lejeune et al. 2018). Hence, future studies are required to decouple the costs and benefits of becoming metamorphic in male Greek smooth newts.

The maintenance of polyphenisms can be driven partially by sexual selection. Non-random mating is a widespread phenomenon in several taxa (Jiang et al. 2013) and contributes to the maintenance of phenotypic diversity within a population (Jaworski et al. 2018). The theory of sexual selection implies, that the abundant sex, females in my case, are competing for access in the opposite sex, males in my case. At the same time, males are becoming choosier, and according to the courtship behavior experiment, it seems they choose female paedomorphs rather than metamorphs.

Empirical studies of the Greek smooth newt (see Chapter 3) and the palmate newt, *Lissotriton helveticus* have shown that there is sexual compatibility between the two heterochronic morphs and little evidence of sexual isolation (Oromi et al. 2016). Similarly, in the alpine newt *Ichthyosaura alpestris*, both homomorphic and heteromorphic encounters were successful, while metamorphic males displayed more to females than paedomorphic ones and no directional choice was found for either morph (Denoël et al. 2001). However, in the Greek smooth newt, metamorphic males displayed less to females than paedomorphs, and there was a directional choice towards paedomorphic females. Female responsiveness was found to be correlated with duration of display rather than male morph. The two morphotypes exhibit different sexual characteristics, with metamorphs having larger cloaca, denser pigmentation, and coloration patterns as in other newt species as well (Denoël et al. 2001). The lack of female choice here, combined with the skewed sex ratios observed in the study population converge to the theory of sexual selection, which may have a crucial role in the maintenance of paedomorphosis in the study population. However, further experiments using already inseminated females and higher male densities are necessary since females might become choosier after first insemination (Gabor & Halliday 1997).

The combined results here suggest that multiple factors are promoting the expression of paedomorphosis in the studied population of the Greek smooth newt. Fitness differences between the two morphotypes in females regarding the reproductive components offer an advantage of becoming a female paedomorph, and especially females benefit from longer residence in the aquatic habitat to gather energy to lay eggs during the breeding period. Although my results only reflect a snapshot of individuals' fitness rather their lifetime reproductive success, they provide useful insight in the understanding of the underlying ecological mechanisms that promote and sustain facultative paedomorphosis. Future studies should account for the genetic profile of individuals and decouple any heterozygosity-fitness correlations that may occur, along with studies accounting for the role of male morphotype in the viability of larvae. Further research should focus in measuring the fitness consequences of each phenotype in each sex during their lifetime and in several developmental stages across generations.

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Chapter 3

Genetic diversity and detection of candidate loci associated with alternative morphotypes in a tailed amphibian

Κεφάλαιο 3

Γενετική ποικιλομορφία και ανίχνευση υποψήφιων τόπων που σχετίζονται με εναλλακτικούς μορφότυπους σε ένα ουρόδηλο αμφίβιο

3.1 Introduction

Most amphibians exhibit complex life cycles, often utilizing diverse and contrasting habitats. Due to their exposure to varying environmental conditions, it is not surprising that phenotypic plasticity in amphibians is widespread (Levis & Pfennig 2019). Phenotypic plasticity offers the flexibility to adapt in heterogeneous environments and decrease intra-population competition by allowing exploiting different niches (Van Buskirk 2002, Berrigan & Scheiner 2004, Miner et al. 2005, Murren et al. 2015).

Facultative paedomorphosis, a widespread polyphenism among several species of salamanders and newts (Wilbur & Collins 1973, Whiteman 1994), is considered to be the result of interactions between ecological factors and genotypes, resulting in the manifestation of alternative morphs according to the costs and benefits experienced in early life stages (Whiteman 1994). Facultative paedomorphic individuals acquire sexual maturity while retaining larval characteristics (e.g., gills) and coexist with metamorphic ones in the same breeding habitat (Denoël et al. 2005). The manifestation of facultative paedomorphosis is thought to be related to fitness differences between the morphs where one morphotype might have greater fitness advantage than the other depending on the prevailing environmental conditions. The ability of an individual to change its phenotype according to environmental cues, specifically to enhance its fitness in the specific habitat (adaptive phenotypic plasticity), can shape the evolution and ecology of the organisms (Nijhout 2003, Moczek et al. 2011). The evolution of paedomorphosis among several species of Urodela arose due to alterations of the mechanisms controlling the metamorphic timing to adapt in changing environmental conditions (Gould 1985). The incidence of paedomorphosis varies among species, populations, and even among sexes in the same breeding habitat, while artificial selection experiments have revealed an additive genetic basis for paedomorphosis (Semlitsch & Wilbur 1989, Voss & Shaffer 1997, Voss et al. 2003). Thus, paedomorphosis can evolve and phenotypic shifts in response to variable environments can be underpinned by genetic differences (Semlitsch & Wilbur 1989, Voss & Shaffer 1997).

Crosses between nearly obligatory paedomorphic and metamorphic salamanders (*Ambystoma mexicanum* and *A. tigrinum*), showed that timing of metamorphosis is regulated by *met1*, a major effect quantitative trait locus (QTL) containing several linked loci associated with brain development and mitochondrial function (Voss 1995, Voss & Shaffer 1997, Voss et al. 2012, Page et al. 2013). However, facultative paedomorphic individuals can metamorphose later in their lifespan in response to alterations in their habitat, such as drought, invasion of alien fish species or crowding conditions (Mathiron et al. 2017, Bohenek & Resetarits 2018, Toli et al. 2020). The mechanism underlying this is thought to be driven by environmental stressors, which regulate the release of thyroid hormones and corticosteroids (Denver 1997, Denver 2017, Bohenek et al. 2021).

Studies of populations of the palmate newt (*Lissotriton helveticus*) exhibiting both morphotypes, showed lack of genetic differentiation among paedomorphic and metamorphic individuals using microsatellites markers (Oromi et al. 2016). The high levels of gene flow between the two morphotypes suggest absence of sexual isolation between them and suggests the persistence of paedomorphosis as a polyphenism in this study population.

Microsatellite markers, although highly polymorphic, have limitations that may limit their power in genetic analysis of populations. Recently, the development of new methods like RAD-sequencing in obtaining single nucleotide polymorphisms (SNPs) has enabled the study of demography, population structure, and adaptation more in depth, with high resolution and accuracy (Väli et al. 2008, Bruneaux et al. 2013, McCartney-Melstad et al. 2018). Especially for species with no prior genetic knowledge or highly complex genomes, like newts, RAD-sequencing is an appealing alternative over other approaches to obtain highly polymorphic markers (Hohenlohe et al. 2011).

Here, I focus on the Greek smooth newt (*Lissotriton graecus*), a small, tailed amphibian found in the southern Balkans, which often exhibits facultative paedomorphosis (Sotiropoulos et al. 2017). By obtaining thousands of SNPs through ddRAD-sequencing, I aimed to 1) detect any genetic structure within the newt population where both morphotypes coexist and high gene flow is expected to occur, 2) explore the genetic diversity of the population as a whole and in respect to each morphotype, 3) detect candidate loci associated with each morphotype and 4) identify candidate genes involved in the expression of the polyphenism. My study provides unique insights in the persistence of the polyphenism by employing population genetic analyses in a single population, thus eliminating the possible association between the morph and the environmental characteristics.

3.2 Materials & Methods

3.2.1 Sample collection and library preparation

A total of 70 smooth newts (17 female metamorphs, 27 female paedomorphs, 11 male metamorphs, 15 male paedomorphs) were sampled during the winter in 2018-2019 from an artificial pond in Ioannina basin, Greece (39°36'N 20°50'E, 497 m.a.s.l). Population and site characteristics have been described in detail in Sotiropoulos et al. (2017). The maturity of captured individuals was verified by a developed cloaca (Denoël 2017) and body size (i.e, individuals larger than 31mm SVL-snout to vent length). Tissue samples from toe clips were collected and preserved in ethanol, and total genomic DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's protocol. ddRAD libraries were produced using an IGATech custom protocol (IGA Technology Services, Udine, Italy), with minor modifications with respect to Peterson's double digest restriction-site associated DNA preparation (Peterson et al. 2012). Genomic DNA was fluorometrically quantified, normalized to a uniform concentration and 300ng were double digested with 2.4U of both *Sph*I (5'GCATG 3') and *Bam*HI (5'GGATCC 3') endonucleases (New England BioLabs) in 30µL reaction supplemented with CutSmart Buffer and incubated at 37°C for 90min, then at 65°C for 20min. Fragmented DNA was purified with 1.5µL of AMPureXP beads (Agencourt) and subsequently ligated with 200U of T4 DNA ligase (New England BioLabs) to 2.5pmol of overhang barcoded adapter for rare cut sites and to 5pmol of overhang barcoded adapter for frequent cut sites in 50µL reaction incubated at 23°C for 60min and at 20°C for 60min followed by 20min at 65°C. Samples were pooled on multiplexing batches and bead purified. For each pool, targeted fragments distribution was collected on BluePippin instrument (Sage Science Inc.) setting the range of 380 bp – 500 bp. Gel eluted fraction was amplified with indexed primers using Phusion High-Fidelity PCR Master Mix (New England BioLabs) in a final volume of 50µL and subjected to the following thermal protocol [95°C, 3min] - [95°C, 30sec - 60°C, 30sec - 72°C, 45sec] x 12 cycles - [72°C, 2min]. Products were purified with 1 volume of AMPureXP beads. The resulting libraries were checked with both Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and Bioanalyzer DNA assay (Agilent Technologies, Santa Clara, CA). Libraries were sequenced with 150 cycles in paired end mode on NovaSeq 6000 instrument following the manufacturer's instructions (Illumina, San Diego, CA).

3.2.2 Quality filtering and variant calling

Raw reads were demultiplexed and trimmed to remove adaptors using the process_radtags utility included in Stacks v2.0 (Catchen et al. 2013). Short reads were de novo assembled, cataloged and matched using *ustacks cstacks, sstacks* and *tsv2bam* (for paired-end reads) utilities in Stacks with default parameters. Single nucleotide polymorphisms were called using *gstacks* which assembles and genotypes contigs. SNP calling was done under the *populations* component included in Stacks, in which only loci that were genotyped in at least 75% of all individuals were kept, with a cut off --max-obs-het=0.8 in order to process a nucleotide site at a locus with observed heterozygosity at maximum of 80%. Further filtering was done using VCFtools v. 0.1.17 (Danecek et al. 2011), in which non-biallelic SNPs were discarded and sites that had a mean read depth between 10x and 90x were kept, resulting in 138,420 SNPs. For downstream populations analysis, a single SNP from each locus was randomly selected using the stacks flag –write_random_snp, resulting in a final set of 27,274 SNPs, with less than 12% missing data per individual (mean=2.5%), less than 7% missing genotypes per SNP (mean=2.5%), and minor allele frequency greater than 1% (Figure 3.1). A final set of 64 individuals were included after the removal of six relatives according to a relatedness matrix, generated with the method proposed by VanRaden (VanRaden 2008) as implemented in the R package snpReady (Granato et al. 2018). Those individuals were removed from downstream analysis since including relatives would lead to biased estimates of parameters such as allele frequencies, expected heterozygosity and F-statistics as well as to spurious population structure (Wang 2018, O'Connell et al. 2019).

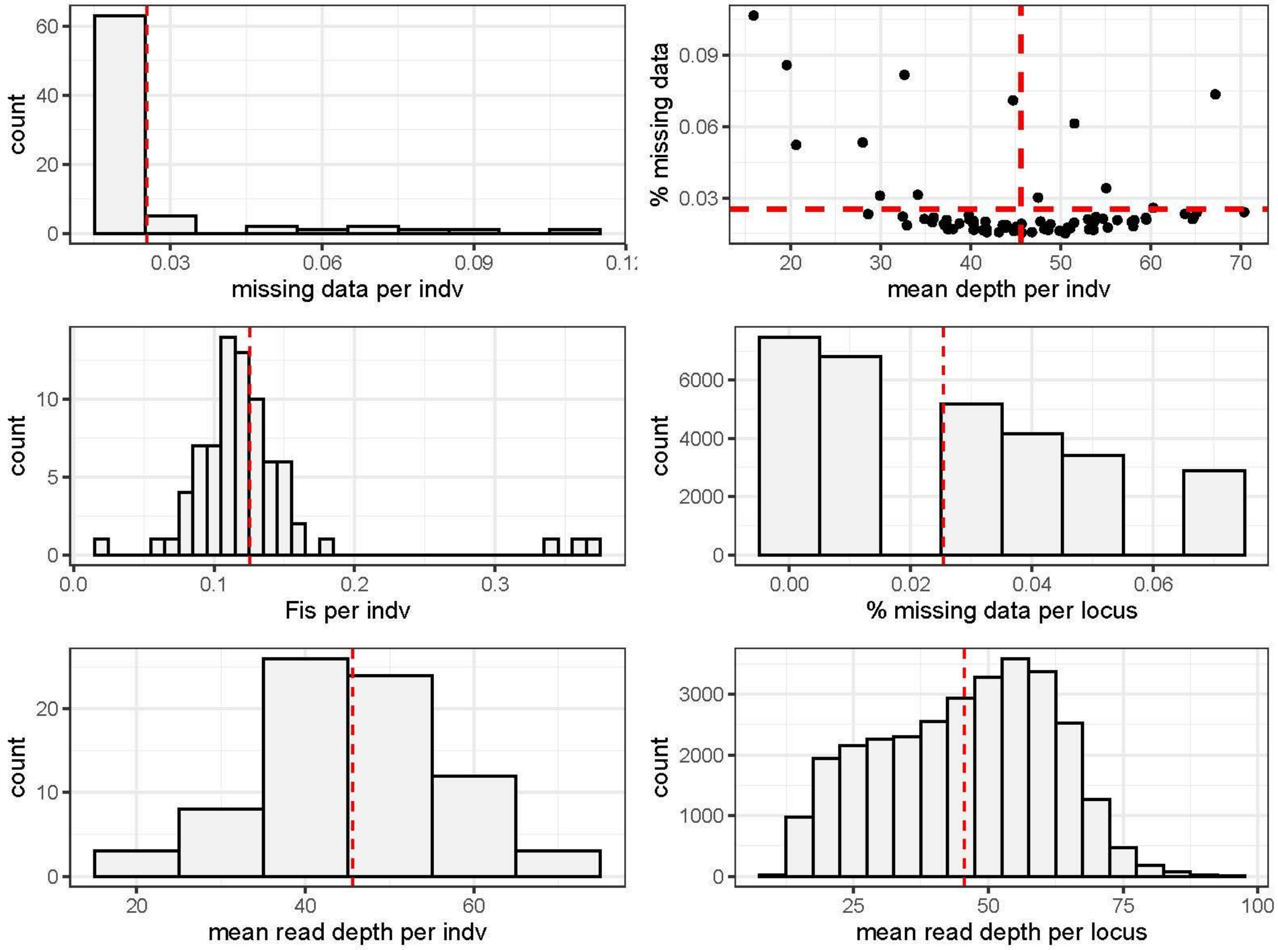

Figure 3.1: Graphical presentation of the final SNP dataset after the filtering procedure. *Fis*: inbreeding coefficient for each individual based on the SNP dataset.

Εικόνα 3.1: Γραφική απεικόνιση του συνόλου των δεδομένων SNP μετά από την τελική διαδικασία φιλτραρίσματος. *Fis*: συντελεστής ενδογαμίας για κάθε άτομο υπολογισμένος από το σύνολο των δεδομένων SNP.

3.2.3 Estimation of genome-wide diversity and structure

Estimates of genomic diversity and differentiation between and within the two morphotypes were calculated in R 3.6.0 (R Core Team 2019), using the packages dartR (Gruber et al. 2018), adegenent (Jombart & Ahmed 2011) and hierfstat (Goudet 2005). Pairwise *Fst* values were obtained by 999 MCMC permutations. Fine-scale population structure within the pond was estimated through a model-based Bayesian clustering method implemented in fineRADstructure v. 0.3 (Lawson et al. 2012). The model was run with a burn-in of 100,000 iterations, 100,000 Markov chain Monte Carlo (MCMC) iterations and a thinning interval of 1,000 iterations. A tree was constructed with 10,000 burn-in iterations and the results were visualized using the scripts fineRADstructurePlot.R (Malinsky et al. 2018).

Lastly, effective population size (N_e) was estimated based on the linkage disequilibrium method as implemented in NeEstimator v. 2 (Do et al. 2014), with minimum allele frequency set to 0.02 and non-parametric jack-knifed confidence intervals (Jones et al. 2016). Since estimates of N_e based on SNP data are usually downwardly biased, Ne estimates were corrected using the equation 1a ($\check{N}_e/N_e=0.098+0.219*\ln(Chr)$) from Waples et al. (2016), where $\check{N}_e$ is the corrected effective size, and *Chr* the haploid number of chromosomes. In *Lissotriton* newts, the haploid number of chromosomes is 12 (Niedzicka et al. 2017).

3.2.4 Outlier analysis

Detection of outlier loci was done using the whole dataset of 138,420 SNPs, under an Fst-based approach implemented in OutFLANK (Whitlock & Lotterhos 2015). OutFLANK infers the distribution of *Fst* for loci that are unlikely to be under selection and then attempts to identify loci that have elevated *Fst* values. The method exhibits lower rates of false positives than most other methods which usually assume a specific model of demographic history (Whitlock and Lotterhos 2015). OutFLANK was run by implementing a left and right trim factor of 0.35 and 0.06, respectively, an FDR of 5 (q threshold = 0.05) and K (number of groups) was set to 2. Allele frequency patterns of each outlier were reported using the PopGenReport (Adamack & Gruber 2014) and tested for significant differences based on morphotype (paedomorphs vs metamorphs) using Fisher's exact test followed by Bonferroni correction for multiple testing. The structure of the detected outlier dataset was explored through a Principal Component Analysis (PCA) implemented in the package dartR. Radtags containing candidate SNPs were used for Blast query (blastn) against the annotated genome of *Ambystoma mexicanum* (AmbMex60DD). Over-representation analysis (ORA) was done using the WebGestalt web server (Liao et al. 2019). The enrichment analysis included biological processes noRedundant, molecular function noRedundant and KEGG pathway (Kyoto Encyclopedia of Genes and Genomes) and was run against the human genome. The GO terms (Gene Ontology terms) with a cut off p-value <0.01, adjusted for multiple testing, were considered over-represented.

3.3 Results

3.3.1 Genomic diversity and structure

A total of 64,975 polymorphic loci were identified and 27,274 SNPs were retrieved after quality filtering for 64 individuals (after the removal of relatives). Both morphotypes (paedomorphic and metamorphic) exhibited similar values of allelic richness and observed heterozygosity (Table 3.1), while no differentiation between the groups was detected (*Fst*=0). Estimates of *Fis* were positive and statistically significant for each group (Table 3.1). FineRADstrucure was unable to infer distinct clusters, indicating there is high gene flow between the two morphotypes (Figure 3.2). The estimate of effective population size, N_e ± (95% CI) was 428.4 ± (295.1-752.4).

Table 3.1: Population summary of genetic statistics in the population and between the two morphotypes. n: number of individuals, *Ar*: Allelic richness, *Ho*: Observed heterozygosity, *He*: Expected heterozygosity, *Pi*: Nucleotide diversity, *Fis*: Fixation index and 95% confidence intervals, *Pa*: Private alleles. Values statistically significant are shown with an asterisk.

Πίνακας 3.1: Σύνοψη γενετικών στατιστικών του πληθυσμού και μεταξύ των δύο μορφότυπων. n: αριθμός των ατόμων, *Ar*: αφθονία αλληλομόρφων, *Ho*: παρατηρούμενη ετεροζυγωτία, *He*: αναμενόμενη ετεροζυγωτία, *Pi*: νουκλεοτιδική ποικιλότητα, *Fis*: συντελεστής ενδογαμίας και 95% όρια εμπιστοσύνης, *Pa*: ιδιωτικά αλληλόμορφα. Οι στατιστικά σημαντικές τιμές δείχνονται με αστερίσκο.

groups	**n**	***Ar***	***Ho***	***He***	***Pi***	***Fis***	***Pa***
paedomorphs	41	1.901	0.221	0.241	0.244	0.085 (0.09-0.10) *	1798
metamorphs	23	1.906	0.220	0.241	0.246	0.086 (0.10-0.11) *	456
population	64	1.99	0.221	0.243	0.245	0.095(0.09-0.10) *	-

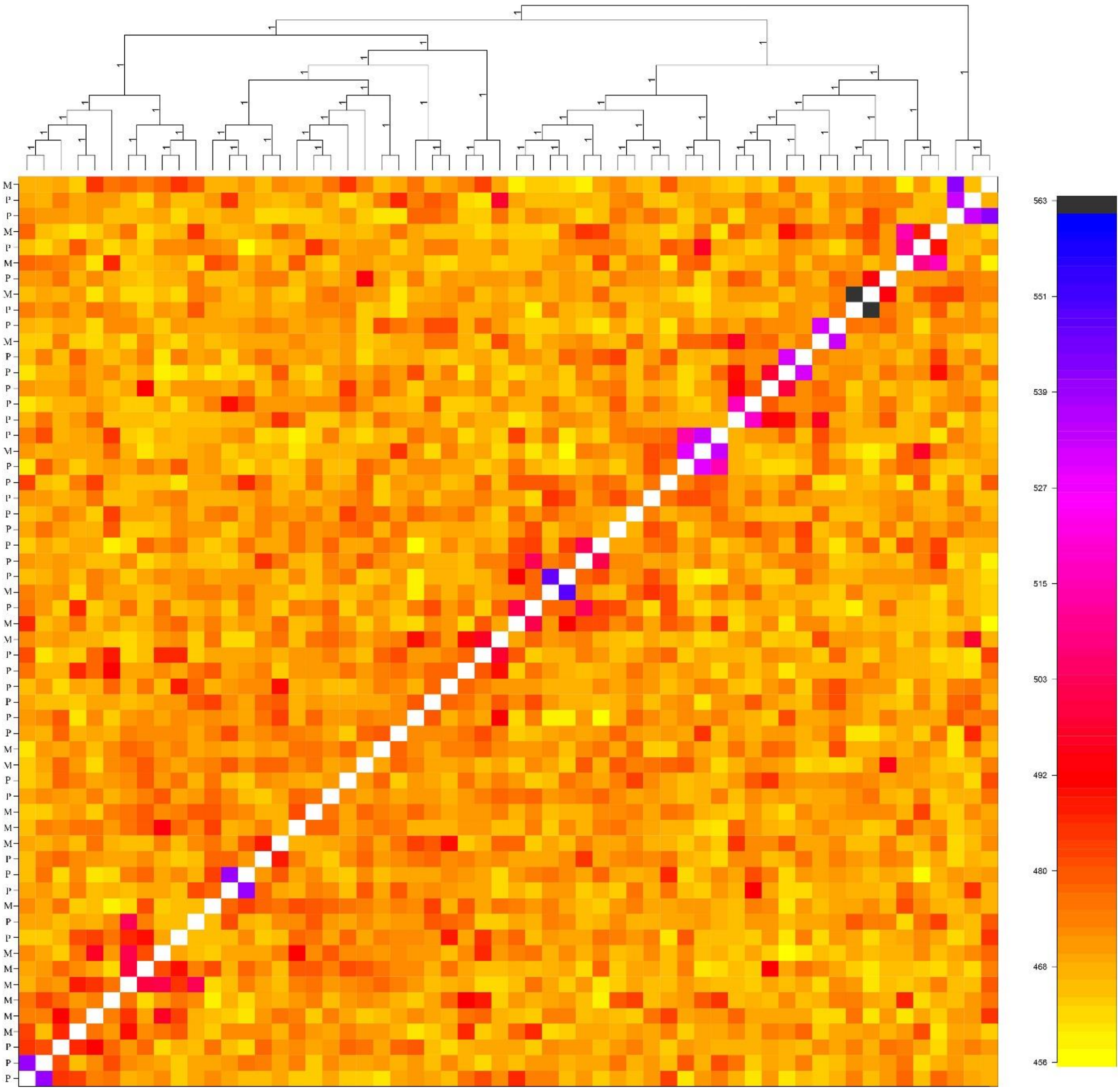

Figure 3.2: Co-ancestry matrix between each pair of individuals generated by fineRADstructure, M: metamorph and P: paedomorph.

Εικόνα 3.2: Πίνακας συγγένειας μεταξύ κάθε ζεύγους ατόμων που δημιουργήθηκε από το fineRADstructure, M: μεταμορφωμένο και P: παιδομορφικό.

3.3.2 Detection of outliers and candidate loci

OutFLANK analysis revealed 35 candidate SNPs (Figures 3.3, 3.4), that are potentially under divergent selection between the two morphotypes in the studied population. Allele frequencies were statistically different in all 35 candidate SNPs between the paedomorphic and metamorphic morph (Table 3.2). The clustering analysis revealed two groups separating the two newt morphotypes (Figure 3.5), with the first two axes explaining more than 60% of the total variation. Pairwise *Fst* was statistically significant (*Fst* = 0.198, p<0.001).

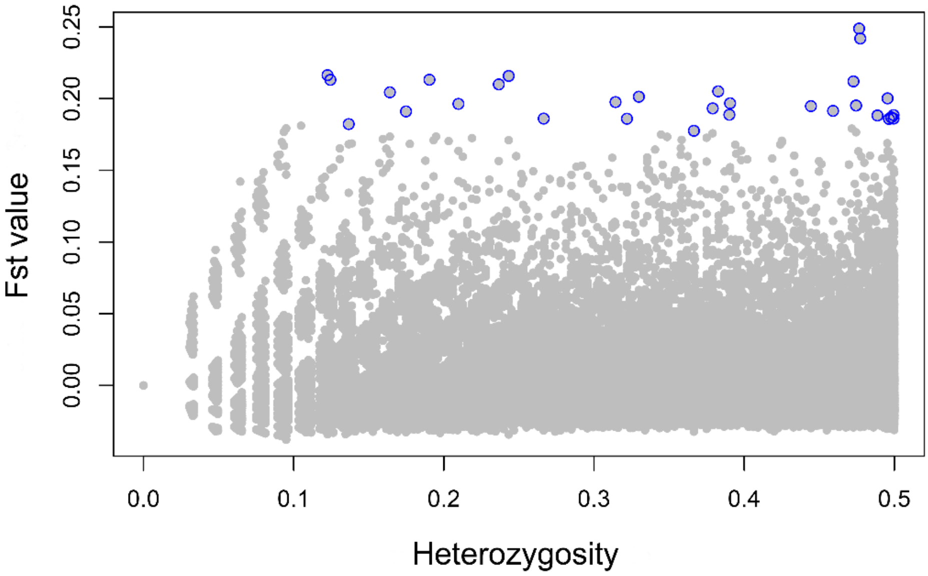

Figure 3.3: Outlier SNPs based on Fst in the OutFLANK analysis. Among 138,420 SNPs, the *Fst* approach identified 35 outliers (in blue). The remaining loci are considered neutral.

Εικόνα 3.3: Ακραίες τιμές SNP που βασίζονται στην ανάλυση OutFLANK. Μεταξύ των 138,420 SNP, η μέθοδος *Fst* εντόπισε 35 ακραίες τιμές (με μπλε). Οι υπόλοιποι γενετικοί τόποι θεωρούνται ουδέτεροι.

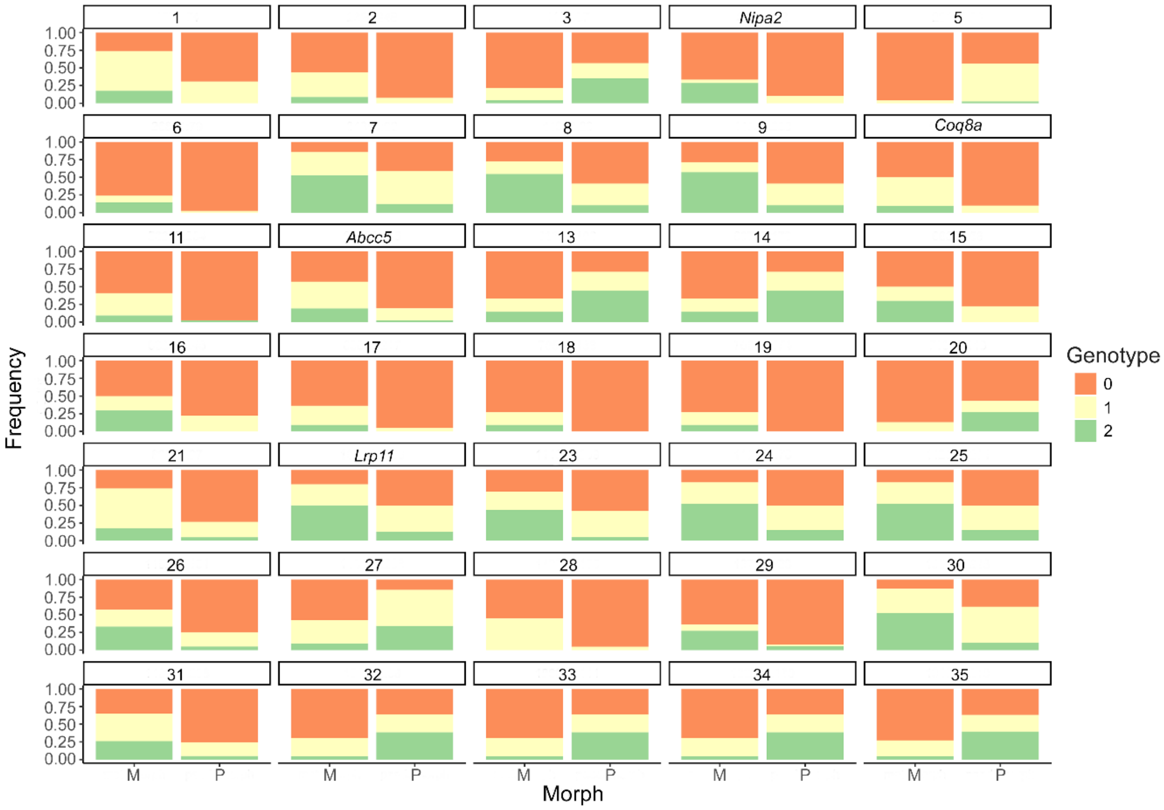

Figure 3.4: Genotype frequencies of the 35 outlier SNPs for each morph (M: metamorphic and P: paedomorphic). 0=homozygous at the reference allele, 1=heterozygous, 2=homozygous at the alternative allele.

Εικόνα 3.4: Γονοτυπικές συχνότητες των 35 γενετικών τόπων για κάθε μορφή (M: μεταμορφωμένο και P: παιδομορφικό). 0=ομόζυγο για το αλληλόμορφο αναφοράς, 1=ετερόζυγο, 2= ομόζυγο για το εναλλακτικό αλληλόμορφο.

Table 3.2: Allele frequencies of major and minor alleles in metamorphic and paedomorphic individuals for each detected outlier. P values are also shown.

Πίνακας 3.2: Συχνότητες αλληλομόρφων σε μεταμορφωμένα και παιδομορφικά άτομα για κάθε ανιχνευθεύντα ακραίο γενετικό τόπο. Παρουσιάζονται και οι τιμές p.

Gene	SNP	Minor Allele	Frequency of minor allele in metamorphs	Frequency of minor allele in paedomorphs	Major Allele	p-value	p-value Bonferroni
1	1653:74	T	0.4565	0.1538	C	0.0003	0.0118
2	2320:148	T	0.2609	0.0375	C	0.0003	0.0120
3	4197:87	A	0.1304	0.4595	G	0.0003	0.0095
Nipa2	9523:172	G	0.3095	0.0513	A	0.0002	0.0075
5	23106:33	G	0.0217	0.2927	C	0.0001	0.0039
6	26834:79	G	0.1905	0.0125	T	0.0008	0.0284
7	34854:90	T	0.6905	0.3537	C	0.0005	0.0194
8	44903:26	A	0.6364	0.2564	G	0.0001	0.0017
9	44903:260	C	0.6429	0.2564	T	0.0001	0.0023
Coq8a	51147:193	T	0.2955	0.0500	G	0.0003	0.0094
11	56367:264	C	0.2500	0.0256	T	0.0002	0.0079
Abcc5	64266:195	T	0.3810	0.1098	C	0.0007	0.0248
13	66737:169	A	0.2381	0.5789	G	0.0005	0.0164
14	66737:179	C	0.2381	0.5789	A	0.0005	0.0164
15	66865:58	C	0.4000	0.1125	G	0.0006	0.0205
16	66865:246	A	0.4000	0.1125	C	0.0006	0.0205
17	69805:217	T	0.2273	0.0250	A	0.0005	0.0186
18	70546:133	A	0.1818	0.0000	G	0.0002	0.0064
19	70546:174	G	0.1818	0.0000	T	0.0002	0.0074
20	71908:116	T	0.0652	0.3514	A	0.0003	0.0117
21	80573:97	A	0.4565	0.1585	G	0.0004	0.0132
Lrp11	106952:166	G	0.6500	0.3125	A	0.0008	0.0265
23	111864:209	A	0.5652	0.2375	T	0.0004	0.0150
24	112861:76	T	0.6739	0.3250	C	0.0002	0.0067
25	112861:264	A	0.6739	0.3250	T	0.0002	0.0067
26	115254:151	C	0.4524	0.1500	T	0.0004	0.0151
27	115510:208	C	0.2619	0.5976	T	0.0006	0.0197
28	118001:155	T	0.2250	0.0244	C	0.0007	0.0244
29	127183:28	T	0.3182	0.0658	A	0.0005	0.0169
30	136600:248	A	0.6957	0.3590	T	0.0004	0.0134
31	149513:206	T	0.4565	0.1463	C	0.0003	0.0088
32	186292:88	G	0.1739	0.5128	A	0.0002	0.0084
33	186292:111	C	0.1739	0.5128	G	0.0002	0.0084
34	186292:180	T	0.1739	0.5128	C	0.0002	0.0084
35	186292:210	G	0.1591	0.5132	A	0.0001	0.0035

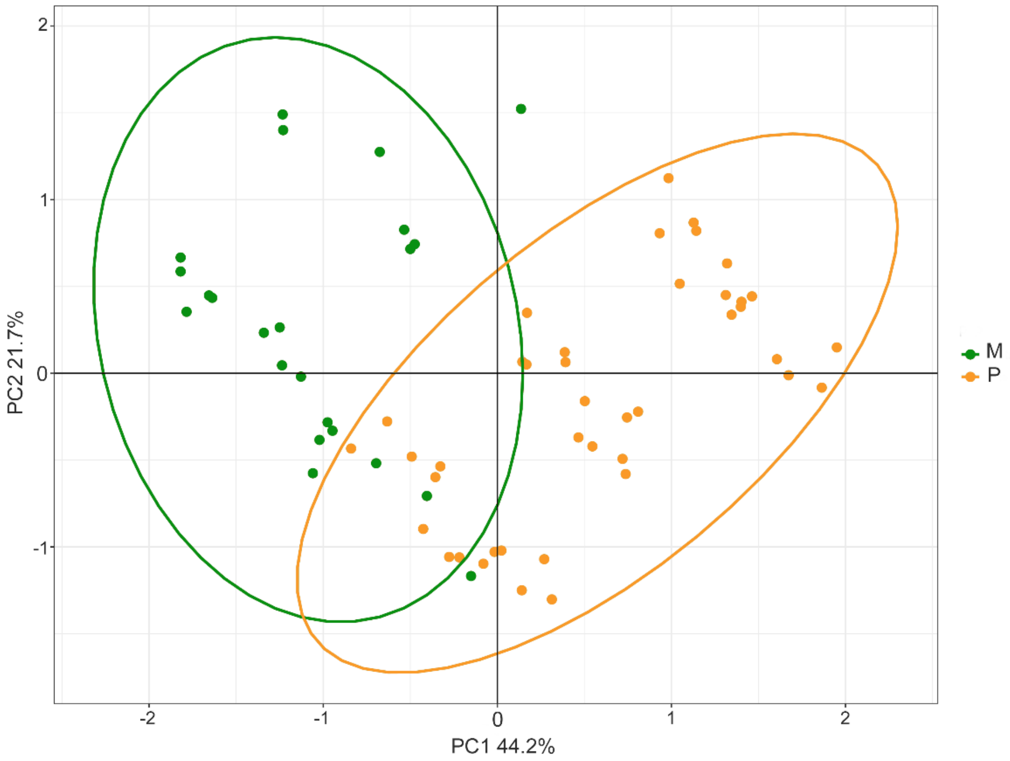

Figure 3.5: PCA plot based on the 35 outlier SNPs and the percent of variance explained in each principal component (M: metamorphic and P: paedomorphic).

Εικόνα 3.5: Διάγραμμα από την ανάλυση κύριων συνιστωσών (PCA) με βάση τις 35 ακραίες τιμές SNP και το ποσοστό διακύμανσης που εξηγείται σε κάθε κύρια συνιστώσα (M: μεταμορφωμένο και P: παιδομορφικό).

Of the detected loci containing SNPs putatively under selection, only four were successfully annotated with a cut-off e-value of 10^{-4} and over 70% identity when Blasted against the Axolotl genome assembly (Table 3.3). The identified genes are associated with metabolic processes and energy production. The top 10 GO terms, obtained for biological processes, cellular component, and molecular function included response to immobilization stress and ADP binding (Figure 3.6, Table 3.4). One significant KEGG pathway involved in antifolate resistance was also identified (Table 3.4).

Table 3.3: Results of gene annotation of the detected outlier loci based on the *Ambystoma mexicanum* assembly (ambMex60DD).

Πίνακας 3.3: Αποτελέσματα της γονιδιακής συσχέτισης/οντολογίας των ανιχνευθέντων γενετικών τόπων με βάση το γονιδίωμα του *Ambystoma mexicanum* (ambMex60DD).

	Axolotl genes	**Gene name**	**Function**
1	*Nipa2*	NIPA Magnesium Transporter 2	Plays an important role in cellular metabolism and energy production reactions
2	*Coq8a*	Coenzyme Q8A	Provides instructions for making coenzyme 10, and plays essential role in oxidative phosphorylation
3	*Abcc5*	ATP Binding Cassette Subfamily C Member 5	Transport various substrates through membranes by utilizing the energy of ATP binding and hydrolysis
4	*Lrp11*	LDL Receptor Related Protein 11	It may be highly correlated with stress related phenotypes

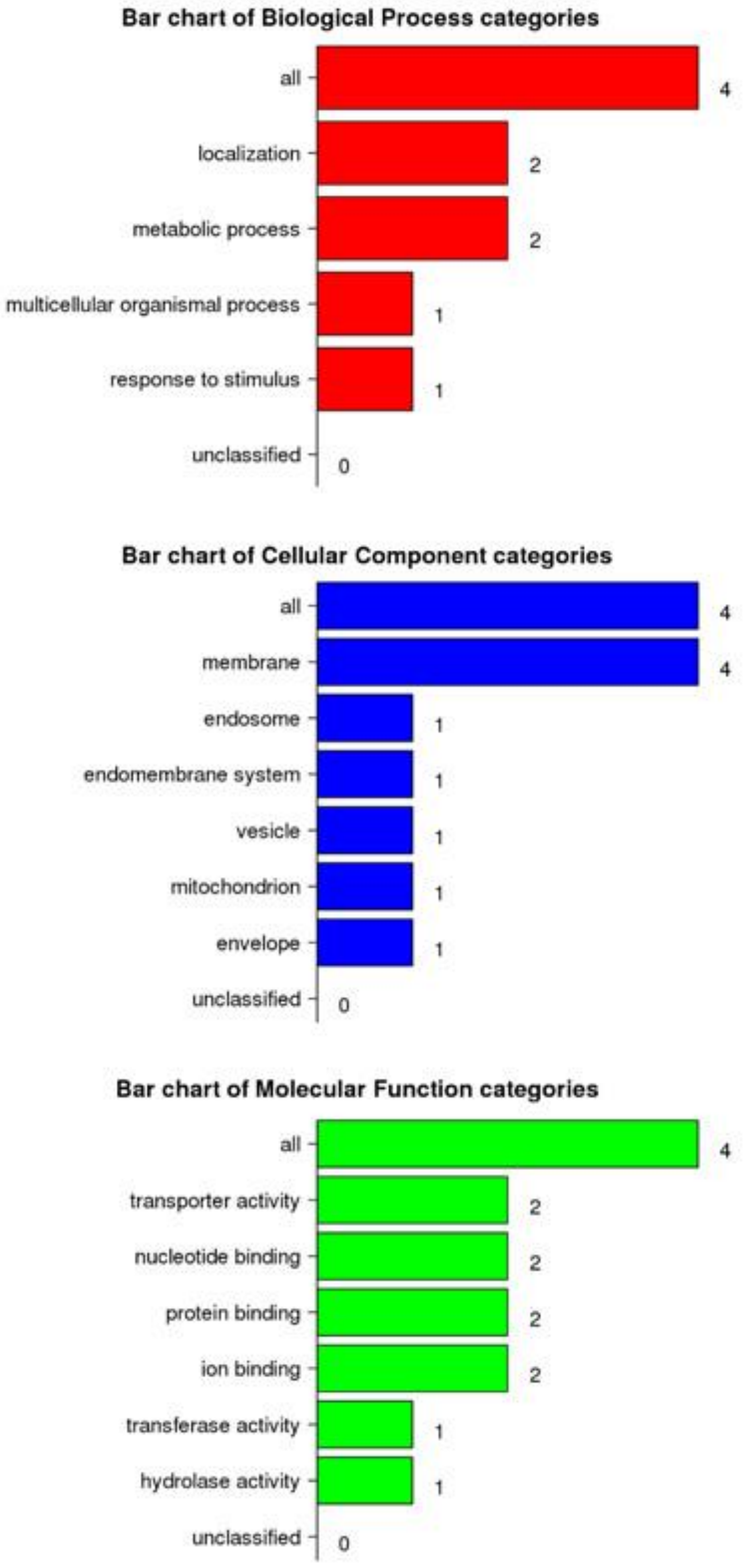

Figure 3.6: GO term analysis by using Go Slim in WebGestalt (Liao et al. 2019). The results indicate that identified genes can be categorized into important biological processes, including localization, metabolic process, membrane, transporter activity, and nucleus, protein binding and ion binding.

Εικόνα 3.6: Ανάλυση GO term χρησιμοποιώντας το GoSlim στο WebGestalt. Τα αποτελέσματα έδειξαν ότι αυτά τα γονίδια συμμετέχουν σε σημαντικές βιολογικές διεργασίες, όπως μεταβολικές διαδικασίες, μεταφορά μορίων και δέσμευση πρωτεϊνών και ιόντων.

Table 3.4: The top 10 GO enrichment categories for the four annotated genes along with the significance statitstics for each category. Enrichment ratio is the number of observed divided by the number of expected genes for each GO category. Expect is the expected number of genes in each GO category. In bold are statistically significant values regarding the cut-off value of 0.01

Πίνακας 3.4: Οι 10 πρώτες κατηγορίες εμπλουτισμού GO για τα τέσσερα γονίδια μαζί με τη στατιστικά σημαντική τιμή για κάθε κατηγορία. Enrichment ratio: ο αριθμός των παρατηρούμενων διαιρεμένος με τον αριθμό των αναμενόμενων γονιδίων για κάθε κατηγορία GO. Expect: ο αναμενόμενος αριθμός γονιδίων σε κάθε κατηγορία GO. Σε έντονη γραφή είναι οι στατιστικά σημαντικές τιμές σύμφωνα με τη τιμή κατώφλι 0.01.

Gene Set	Description	Number of genes	Enrichment ratio	Expect	P value
GO:0035902	Response to immobilization stress	27	152.12	0.00657	**0.00655**
hsa01523	Antifolate resistance	31	132.49	0.00754	**0.00752**
GO:0043531	ADP binding	39	105.31	0.00949	**0.00946**
hsa02010	ABC transporters	44	93.347	0.01071	0.01067
GO:0033555	Multicellular organismal response to stress	74	55.503	0.01801	0.01789
GO:0051219	Phosphoprotein binding	81	50.707	0.01972	0.01957
GO:0006022	Aminoglycan metabolic process	165	24.892	0.04017	0.03957
GO:0015698	Inorganic anion transport	177	23.205	0.04309	0.0424
GO:0042180	Cellular ketone metabolic process	184	22.322	0.04479	0.04405
GO:0009266	Response to temperature stimulus	201	20.434	0.04893	0.04805

3.4 Discussion

My findings corroborate previous studies showing high gene flow between the alternative phenotypes that coincides with sexual compatibility between the two morphs (Oromi et al. 2016). The lack of strong genetic differentiation suggests that facultative paedomorphosis remains as a polyphenism in the study population. Each morph exhibited similar levels of observed heterozygosity, which were lower than expected suggesting inbreeding. Inbreeding leads to loss of gene diversity and thereby also to loss of adaptive variation. Although many populations seem to thrive under low levels of diversity, it is a risk factor that can compromise adaptation in changing environments (Hedrick & Kalinowski 2000, Linløkken 2018).

The coexistence of both morphotypes in the same breeding habitat could be explained by fitness advantages favoring each morph and a way to lessen intra-specific competition through ecological niche partitioning (e.g., trophic resources, shelter; Lejeune et al. 2018). In early life stages, larvae receive the environmental cues and information of the surroundings and assess which phenotype will be associated with higher fitness (Whiteman 1994). Switching habitats and going through metamorphosis is time and energy consuming, and individuals may avoid the cost and gain long-term advantages by remaining paedomorphic (Denoël et al. 2002). On the other hand, metamorphic individuals can disperse on land, harness both aquatic and terrestrial resources and avoid competition (Denoël et al. 2005). The assessment, based on environmental information, would be followed by a phenotypic response, which requires the employment of several complex physiological and behavioral mechanisms (Beldade et al. 2011).

The identified candidate genes are involved in metabolic processes and stress response. *Nipa2* gene encodes the transportation of Mg^{++} in cytoplasm, which is highly important for production of energy and proteins in cells (Goytain et al. 2008), as well the Coenzyme 10, encoded by the *Coq8a* gene, which converts energy from food in a useful form utilized by cells in a process called oxidative phosphorylation (Reidenbach et al. 2018). Phenotypic shifts are indeed characterized by high energy consumptions and metabolic changes.

Although larvae make the decision to either become paedomorphic or metamorphic in early larval stages (Whiteman et al. 2012), in the case of facultative paedomorphosis, newts can metamorphose at any point of their life span in the presence of environmental stressors (Mathiron et al. 2017, Toli et al. 2020). In stressful conditions, such as pond drying, the presence of alien fish species that act as predators, as well as limited trophic resources (Mathiron et al. 2017, Lejeune et al. 2018, Toli et al. 2020), adult paedomorphic newts may transition to the terrestrial form, thus avoiding such hostile aquatic conditions and enhance their fitness through this adaptive change. Stress hormones have been the focus of amphibian metamorphosis with respect to thyroid hormone which regulates metabolic processes and plays a key role in the initiation of metamorphosis (Tata 2006, Voss et al. 2012). Stress response is controlled by many genes and the *Lrp11* gene, identified in the present study as an outlier, has been associated with stress related phenotypes and responses (Xu et al. 2014).

Furthermore, the observed overrepresented GO terms related to stress response and ADP binding, highlight the importance of stress and energy in phenotypic shifts. ADP is an important molecule which is converted to ATP, the energy form used by cells for many enzymatic reactions, transportation of hormones, neurotransmitters, and other molecules (Wang & Tajkhorshid 2008). More interestingly, the antifolate resistance pathway describes how cells may develop resistance to antifolates, which act as antagonists of folic acid. It has been demonstrated that antifolates inhibit growth rates in amphibian larvae (Rosenbaum & Velardo 1951). The genes involved in this pathway may contribute to the development to each specific morph and regulate the growth rate and size of paedomorphic individuals.

Although a growing number of genomes for non-model organisms are becoming available, the lack of a reference genome here is hindering a further in-depth exploration of my data. Especially, in traits like facultative paedomorphosis, that are proposed to be polygenic traits controlled by multiple genes dependent from the environment (Harris 1987, Voss 1995, Crowner et al. 2019), it would be difficult to detect any signals of strong selection, especially in the presence of high gene flow (Yeaman 2015).

These results offer insights into facultative paedomorphosis in newts and suggest that this trait remained as a polyphenism in the study population. Based on the SNP dataset, I was able to identify candidate genes associated with each morphotype and their involvement in metabolic processes. Additional studies validating the candidate loci associated with the two morphs in other populations (and other newt species) exhibiting facultative paedomorphosis would be desirable, while future research in more ecologically diverse scales will help determining the underlying mechanisms that mediate plasticity in newts.

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Chapter 4

Genetic insight into a polygenic trait using a Genome Wide Association analysis in a wild amphibian population

Κεφάλαιο 4

Γενετική θεώρηση ενός πολυγονιδιακού χαρακτηριστικού με τη χρήση μιας Ανάλυσης Συσχέτισης Ευρέος Γονιδιώματος σε έναν άγριο πληθυσμό αμφίβιων

4.1 Introduction

Growth related traits are of great importance in amphibians since many fitness traits have been identified to correlate with body growth. Body size varies in amphibians, while different sizes offer both advantages and disadvantages depending on the environment (Levy & Heald 2016). Smaller sizes may offer better chances in escaping predators and/or exploit different food resources, however on the other hand larger sizes may have fewer predators and control temperature and hydration more efficiently (Levy & Heald 2016).

Amphibians exhibit complex life cycles, while changes in body size and growth, by heterochronic processes, often lead to morphological variations like the retention of gills in the adult stage (Denoël & Ficetola 2014). Many amphibian populations and specifically newts and salamanders, exhibit facultative paedomorphosis where paedomorphic individuals coexist with metamorphic ones in the same breeding habitat (Whiteman 1994, Denoël et al. 2005). Reports have shown sex specific and morph specific differences in body size in several newt populations, depending on the environment along with life history traits, and the long-term advantages to opt for bigger or smaller body sizes (Denoël et al. 2002, Denoël et al. 2009, Lejuene et al. 2021). Variation in body size is associated with variation in other life history traits like fecundity and survival. Fecundity has been positively correlated with clutch size and egg mass, hence an increased body size has been favored (Stearns 1992). For example, several studies have conformed the positive relationship between body size and fecundity in various amphibian populations (Semlitsch 1985, Nobili & Accordi 1997, Camargo et al. 2005, Green 2015).

Life-history and growth traits, like body size, are usually affected by many loci, and the development of new methods in obtaining thousands of marker loci has enabled the study of genome-wide diversity, and genotype-phenotype associations even in complex genomes (Bush & Moore 2012). Genome Wide Association studies (GWAs) have greatly facilitated the identification of genes that contribute to differences in fitness and elucidate genomic regions associated with complex phenotypic traits (Husby et al. 2015, Duntsch et al. 2020, Tietgen et al. 2021). The application of GWAs have been primarily focused on model species or economically important crops and aquaculture (Chen et al. 2018, Li et al. 2018, Li et al. 2020). One promising technology that enables the study of non-model species where no reference genome is available, is the double digest RAD sequencing approach (dd-RAD) which reduces the complexity of genomes and identify thousands of single nucleotide polymorphisms, thus enabling GWAs approaches (Peterson et al. 2012).

Body size is a quantitative trait, likely affected by many loci, hence heterozygosity-fitness correlations (HFCs) may occur (Szulkin et al. 2010). Although, the detection of such correlations is usually difficult due to marker loci not representing genome-wide heterozygosity or the lack of variation in inbreeding (Hansson & Westerberg 2002). However, HFCs may arise due to local effects from fitness-related loci linked to markers used to estimate diversity (Szulkin et al. 2010). Loci that may have a functional role or are in vicinity with true functional loci, may have greater heterozygosity effect on fitness traits, either positive or negative depending on the underlying genetic model, than genome-wide heterozygosity (David 1997, Lieutenant-Gosselin & Bernatchez 2006, Bateson et al. 2016).

In this study I focus on a facultative paedomorphic *Lissotriton graecus* population. In this population, paedomorphic females have on average larger body size and better body condition than metamorphic females, although no differences were found between the two morphotypes in males (see Chapter 1). I conducted a fitness experiment, where several reproductive fitness proxies were measured, including the number of eggs laid and the survival of larvae from both paedomorphic and metamorphic female newts. I found a positive relationship of fecundity with body size and differences between the morphs in the reproductive components (see Chapter 1). By obtaining thousands of marker loci through dd-RAD sequencing I aim to 1) detect possible marker loci that are associated with the variation in body size, 2) detect possible heterozygosity-fitness correlations, 3) investigate whether variation in the body size-associated loci, measured as multilocus heterozygosity, is correlated with reproductive fitness components, and lastly 4) identify possible candidate genes and possible morph-specific differences in the associated marker loci.

4.2 Materials & Methods

4.2.1 SNP Genotyping and calling

A total of 39 female (25 paedomorphs and 14 metamorphs) and 24 (14 paedomorphs and 10 metamorphs) male newts were captured from the studied population using dip-nets. Briefly, total genomic DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's protocol. dd-RAD libraries were produced using an IGATech custom protocol (IGA Technology Services, Udine, Italy), with minor modifications with respect to Peterson's double digest restriction-site associated DNA preparation (Peterson et al. 2012). Libraries were sequenced with 150 cycles in paired end mode on NovaSeq 6000 instrument following the manufacturer's instructions (Illumina, San Diego, CA). Raw reads were demultiplexed and trimmed to remove adaptors using the process_radtags utility included in Stacks v2.0 (Catchen et al. 2013). Short reads were *de novo* assembled, cataloged and matched using *ustacks cstacks, sstacks* and *tsv2bam* (for paired- end reads) utilities in the denovo pipeline in Stacks using default parameters. Single nucleotide polymorphisms were called using *gstacks* which assembles and genotypes contigs. SNP calling was done under the *populations* component included in Stacks, in which only loci that were genotyped in at least 75% of all individuals were kept, with a cut off --max-obs-het=0.8 in order to process a nucleotide site at a locus with observed heterozygosity at maximum of 80% Further filtering was done using VCFtools v. 0.1.17 (Danecek et al. 2011), in which non-biallelic SNPs were discarded and sites that had a mean read depth between 10x and 90x were kept. For downstream analysis, a single SNP from each locus was randomly selected using the stacks flag –write_random_snp, with minor allele frequency MAF>0.05, while markers deviating from Hardy-Weinberg equilibrium were discarded.

4.2.2 Phenotypic traits

As fitness traits I considered Snout to Vent Length (SVL) and reproductive fitness components for female newts (number of laid eggs, number of successfully hatched eggs and number of survived larvae till sub-adult stage). Fitness traits were measured during a fitness experiment described in detail in Chapter 1.

4.2.3 Identification of trait-associated loci

An association analysis was done only for females due to the small sample size of males and high missing data in measurements of phenotypic traits of males. Further filtering was done to get the final dataset and remove possible relatives based on a relatedness matrix, generated according to the method proposed by VanRaden (VanRaden 2008) implemented in the R package snpReady (Granato et al. 2018). Association analysis between the genotypic dataset from the polymorphic SNPs and the measured phenotypic traits was done in TASSEL (Bradbury et al. 2017), using a General Linear Model (GLM). Structure analysis (see Chapter 3) inferred a single gene pool with no differentiation between the two morphotypes. Morphotype (paedomorphic vs metamorphic) was added as fixed factor in the model due to morph-specific differences in the phenotypic traits (see Chapter 2). SVL was log-transformed while square root transformations were done for the count data of female reproductive components (number of eggs, number of successfully hatched eggs and number of survived larvae). Model fit was evaluated by Q-Q plots and significant values were corrected for multiple testing. Sequence tags containing significant trait-associated SNPs were used for Blast query against the annotated genome of *Ambystoma mexicanum* (AmbMex60DD), while Over-Representation Analysis (ORA) was done using the WebGestalt web server (Liao et al. 2019). The enrichment analysis included biological processes noRedundant, molecular function noRedundant and KEGG pathway (Kyoto Encyclopedia of Genes and Genomes) and was run against the human genome. The GO terms (Gene Ontology) with a cut off p-value <0.01, adjusted for multiple testing, were considered as over-represented.

4.2.4 Heterozygosity-fitness correlations

Standardized individual multilocus heterozygosity values (sMLH) were calculated based on the genome-wide SNP dataset containing neutral loci for every individual and for the dataset containing the trait-associated loci for female newts in the inbreedR package (Stoffel et al. 2016). sMLH is calculated as the ratio of individual heterozygous loci to the mean number of heterozygous loci in the population typed in the individual, thus avoiding biases due to differences in typed loci between the individuals (Coltman et al. 1999). Linear and generalized models were used to analyze the correlation between levels of individual heterozygosity and the fitness-related traits in R (R Core Team 2021). Log(SVL) was fitted in a model using heterozygosity coefficients, morph and sex as fixed factors. Generalized linear models were also fitted for the number of laid eggs, the number of successfully hatched eggs, and the number of survived larvae. Morph, heterozygosity coefficients and log(SVL) were included as fixed-effect factors. Since these traits are count data, models were tested for overdispersion, using a negative-binomial error distribution implemented in the R package MASS (Venables & Ripley 2002).

4.2.5 Fitness analysis of candidate genes

For each marker loci that was found to be putatively associated with fitness traits and was successfully annotated, patterns of allele and genotype frequencies were observed between the two morphotypes. Frequency of both alleles in each morphotype was determined and tested for significant differences using a Fisher's exact test in Plink v.1.9 (Chang et al. 2015). The relationship of the genotypes of each identified gene and body size was evaluated with general linear model using morph as a fixed effect. Log(SVL) was fitted as the response variable while each locus was fitted as a three-level factor (homozygous at the reference allele, heterozygous and homozygous at the alternative allele), and differences were evaluated by Anova table.

4.3 Results

4.3.1. Phenotypic traits

A total of 64,975 polymorphic loci were generated, and after quality filtering the final dataset contained 19,104 polymorphic loci for a total of 63 individuals. Mean values of the recorded phenotypic traits for paedomorphic and metamorphic female and male newts and differences between them are summarized in detail in Chapter 2. Paedomorphic females exhibited larger SVL and body condition from metamorphic females (Chapter 2). Metamorphic individuals laid more eggs and had a higher number of successfully hatched eggs than paedomorphs (Chapter 2). No statistically significant differences in body size between the two morphotypes were found in male newts (Chapter 2).

4.3.2 Trait-associated loci

GWAS analysis was done on 39 female newts based on 19,080 SNPs after quality filtering and removal of relatives, revealing significant associations with the phenotypic trait of log(SVL). I did not perform an association analysis on males due to the smaller sample size and higher number of missing values on phenotypic traits. A total of 37 loci were identified as putatively associated loci, after adjustment of p values using the false discovery rate (FDR) for multiple testing (Table 4.1, Figure 4.1). The highest percentage of phenotypic variation explained is attributed to marker 73765:235 (0.433). No significant marker associations were found on reproductive components. Blast analysis was performed for all the suggestive associated SNPs revealed by the genome-wide association analysis. Four of the Rad-tags containing the significant SNPs were successfully annotated against the genome of *A. mexicanum*, with over 70% identity and a cut-off e-value of 10^{-4} (Table 4.2).

Table 4.1: Significant marker effect (F) and phenotypic variation explained (R^2) by each associated SNP as revealed by the GLM model in Tassel.

Πίνακας 4.1: Σημαντική επίδραση του δείκτη (F) και φαινοτυπική διακύμανση που εξηγείται (R^2) από κάθε σχετιζόμενο SNP, όπως προέκυψε από το μοντέλο GLM στο Tassel.

Trait log(SVL)		Marker effects GLM		
	SNP ID	F	P-value	R^2
1	44330:271	41.781	1.59E-09	0.432
2	73717:59	46.086	1.58E-07	0.391
3	170820:203	43.101	2.14E-07	0.362

4	39403:269	43.635	2.22E-07	0.373
5	73765:235	24.056	8.32E-07	0.433
6	49686:155	20.062	2.25E-06	0.354
7	79772:241	31.476	4.16E-06	0.365
8	67800:201	18.802	4.49E-06	0.357
9	46720:240	18.630	4.86E-06	0.352
10	137867:161	18.829	5.04E-06	0.355
11	37792:162	18.981	6.17E-06	0.380
12	45028:196	17.484	8.25E-06	0.342
13	66584:6	27.987	9.34E-06	0.312
14	62502:199	17.383	9.69E-06	0.322
15	133872:154	25.231	1.72E-05	0.276
16	31758:260	15.645	2.01E-05	0.327
17	10344:104	15.671	2.43E-05	0.275
18	101108:230	14.998	2.54E-05	0.308
19	3152:121	14.317	3.62E-05	0.301
20	126084:166	14.298	3.66E-05	0.300
21	34901:127	14.781	3.75E-05	0.310
22	61183:132	14.205	3.84E-05	0.299
23	85990:131	13.959	4.38E-05	0.297
24	31018:211	13.930	4.45E-05	0.296
25	149598:42	14.197	4.58E-05	0.318
26	99961:14	14.136	4.73E-05	0.312
27	147756:47	13.624	5.24E-05	0.293
28	18342:221	13.624	5.24E-05	0.293
29	102168:186	14.063	5.38E-05	0.299
30	68091:201	13.462	5.72E-05	0.291
31	36410:57	13.674	6.01E-05	0.311
33	71107:186	13.448	6.24E-05	0.299
33	119718:133	13.186	6.65E-05	0.287
34	645293:118	13.281	6.82E-05	0.291
35	135740:14	13.045	7.19E-05	0.286
36	89138:252	20.119	8.80E-05	0.249
37	73726:170	12.694	9.39E-05	0.284

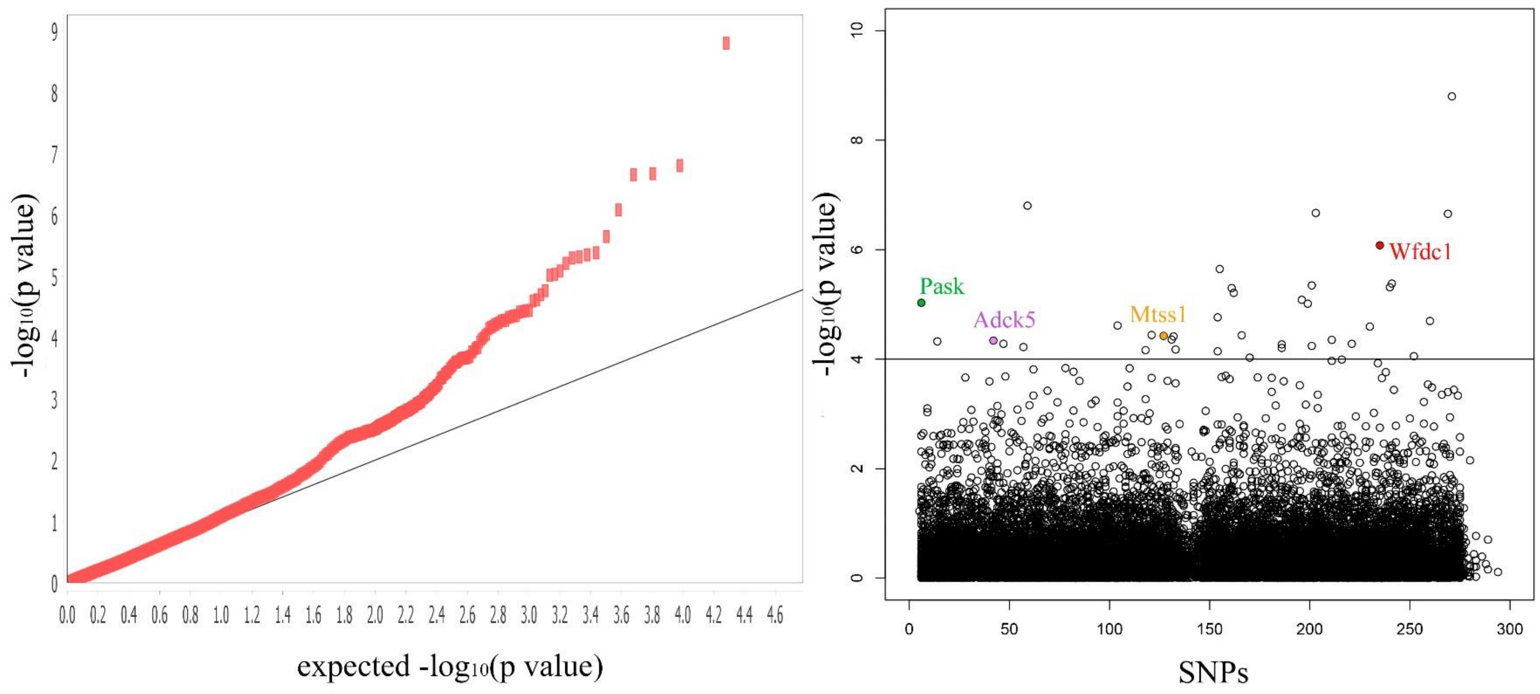

Figure 4.1: Quantile-quantile (Q-Q) plot and Manhattan plot of genome-wide association for the phenotypic trait of logSVL. Most p values were similar to the expected diagonal indicating the good fit of the GWAS model. Line in the Manhattan plot corresponds to the significant threshold after correcting for multiple testing. Genes that were successfully annotated are shown.

Εικόνα 4.1: Q-Q και Manhattan γραφήματα της ανάλυσης συσχέτισης γονιδιώματος με το σωματικό μέγεθος. Οι τιμές p φαίνεται να ακολουθούν κανονική κατανομή υποστηρίζοντας το μοντέλο GWAS. Η ευθεία γραμμή στο γράφημα Manhattan αντικατοπτρίζει το κατώφλι σημαντικότητας. Παρουσιάζονται επίσης και τα γονίδια που χαρτογραφήθηκαν επιτυχώς στην παρούσα μελέτη.

Table 4.2: List of candidate genes identified in the present study and their functions. % Identity and E value are presented according to the Blast results.

Πίνακας 4.2: Τα γονίδια που χαρτογραφήθηκαν στην παρούσα μελέτη. % Identity και E value παρουσιάζονται σύμφωνα με την ανάλυση Blast.

	Gene	**Gene Name**	**Function**	**Associated Trait**	**% Identity**	**E value**
1	*Adck5*	AarF Domain Containing Kinase 5	Its function is not clear yet. It may have a similar function with the Adck1, in maintaining mitochondrial cristae formation and mitochondrial function	log(SVL)	85.37	6e-16
2	*Pask*	PAS Domain Containing Serine/Threonine Kinase	Expression of this gene is regulated by glucose, and the encoded protein may have a role in the regulation of insulin gene expression. May also participate in respiratory regulation.	log(SVL)	78.87	1e-04
3	*Mtss1*	MTSS I-BAR Domain Containing 1	It may be involved in cellular response to fluid shear stress and regulation of cell proliferation	log(SVL)	72.03	4e-05
4	*Wfdc1*	WAP Four-Disulfide Core Domain 1	It encodes proteins that function as growth inhibitors	log(SVL)	79.55	6e-09

Of the identified genes, the *Wdfc1* explained the 43% of total variation in the phenotypic trait of body size (Table 4.1). The identified genes are associated with metabolic processes, biological regulation, and response to stimulus. The top 10 GO terms, obtained for biological processes, cellular component, and molecular function included epithelial cell proliferation, protein serine/threonine kinase activity, and response to fluid shear stress (Table 4.3).

Table 4.3: GO enrichment categories for the four genes for the log(SVL) trait along with the statistically significant value for each category. 'Enrichment ratio' is the number of observed divided by the number of expected genes for each GO category. 'Expect' is the expected number of genes in each GO category. P-values with asterisk are statistically significant regarding the cut-off value 0.01.

Πίνακας 4.3: Οι 10 πρώτες κατηγορίες εμπλουτισμού GO για τα τέσσερα γονίδια μαζί με τη στατιστικά σημαντική τιμή για κάθε κατηγορία. Enrichment ratio: ο αριθμός των παρατηρούμενων διαιρεμένος με τον αριθμό των αναμενόμενων γονιδίων για κάθε κατηγορία GO. Expect: ο αναμενόμενος αριθμός γονιδίων σε κάθε κατηγορία GO. Σε έντονη γραφή είναι οι στατιστικά σημαντικές τιμές σύμφωνα με τη τιμή κατώφλι 0.01.

Gene set	**Description**	**Size**	**Expect**	**Ratio**	**P value**
GO:0050673	Epithelial cell proliferation	372	0.091	22.082	0.003*
GO:0004674	Protein serine/threonine kinase activity	449	0.109	18.295	0.004*
GO:0034405	Response to fluid shear stress	33	0.008	124.46	0.008*
GO:0007585	Respiratory gaseous exchange	64	0.016	64.176	0.015
GO:0005976	Polysaccharide metabolic process	107	0.026	38.386	0.026
GO:0032355	Response to estradiol	126	0.031	32.597	0.030
GO:1903034	Regulation of response to wounding	152	0.037	27.021	0.036
GO:0006109	Regulation of carbohydrate metabolic process	161	0.039	25.511	0.039
GO:0031348	Negative regulation of defense response	187	0.046	21.964	0.045
GO:0016051	Carbohydrate biosynthetic process	205	0.049	20.035	0.049

4.3.3 Heterozygosity-fitness correlations

Values of standardized multi-locus heterozygosity (sMLH) regarding the genome-wide dataset for both sexes were ranging from 0.923 to 1.109, with a mean ±SD: 1.001±0.028. Genome-wide heterozygosity was not correlated with any of the phenotypic traits, although morph and sex had a significant effect on log(SVL) (Table 4.4). Regarding the trait-associated dataset for female newts, values of sMLH were ranging from 0.244 to 1.864, with a mean ±SD: 0.952± 0.386. No significant effects of heterozygosity were found on any reproductive fitness components (Table 4.4).

Table 4.4: Estimates of fixed effects in each model. Significant values are shown in bold.

Πίνακας 4.4: Αποτελέσματα γραμμικών μοντέλων. Οι στατιστικά σημαντικές τιμές είναι σε έντονη γραφή.

Log(SVL)	**Estimate**	**Std. Error**	**T value**	**P value**
(Intercept)	1.340	0.244	5.477	**<0.001**
sMLH	0.176	0.244	0.725	0.471
Morph (paedomorph)	0.031	0.015	2.069	**0.043**
Sex (male)	-0.031	0.015	-2.023	**0.047**
Number of eggs				
(Intercept)	7.9133	7.572	1.045	0.305
sMLH	-6.134	4.658	-1.317	0.198
sMLH trait associated	-0.051	0.359	-0.141	0.889
Morph (paedomorph)	-0.729	0.467	-1.559	0.130
Log(SVL)	2.088	4.158	0.502	0.619
Number of hatched eggs				
(Intercept)	12.005	8.722	1.376	0.179
sMLH	-8.317	5.371	-1.548	0.132
sMLH trait associated	-0.057	0.413	-0.138	0.891
Morph (paedomorph)	-0.895	0.538	-1.663	0.107
Log(SVL)	0.562	4.784	0.118	0.907
Number of surviving larvae till the sub-adult stage				
(Intercept)	-8.375	5.708	-1.467	0.142
sMLH	-2.168	2.926	-0.741	0.458
sMLH trait associated	0.043	0.256	0.170	0.864
Morph (paedomorph)	-0.260	0.347	-0.749	0.453
Log(SVL)	7.509	3.325	2.258	**0.023**

4.3.4 Fitness analysis of candidate genes

Allele frequencies of the four QTLs were not statistically different between the two morphotypes (Figure 4.2). However, morph specific genotype differences were observed (F=63.83, d.f.=1, p<0.001). Variants in genes *Wfdc1* and *Pask* had a significant effect in female paedomorphic newts, (F=10.867, d.f.=2, p=0.001 and F=8.815, d.f.=1, p=0.009, respectively). Carriers of the G allele in *Wfdc1* gene, had smaller body size compared to the homozygous (TT) in paedomorphic females (Figure 4.3), while in metamorphic females only homozygous (TT) were found (Figure 4.3). We found no individuals heterozygous to the alternative allele (CC) at *Pask* gene, however both morphotypes exhibited larger body size when they were homozygous at the reference allele (AA) in respect to the heterozygous individuals (AC) (Figure 4.3). Variants in gene *Adck5* had a significant effect in female metamorphic newts (F=7.221, d.f.=1, p=0.027). Interestingly, metamorphic individuals homozygous for the alternative G allele in *Adck5* had a significant lower log(SVL), while frequency of homozygous paedomorphic was very low (Figures 4.2, 4.3).

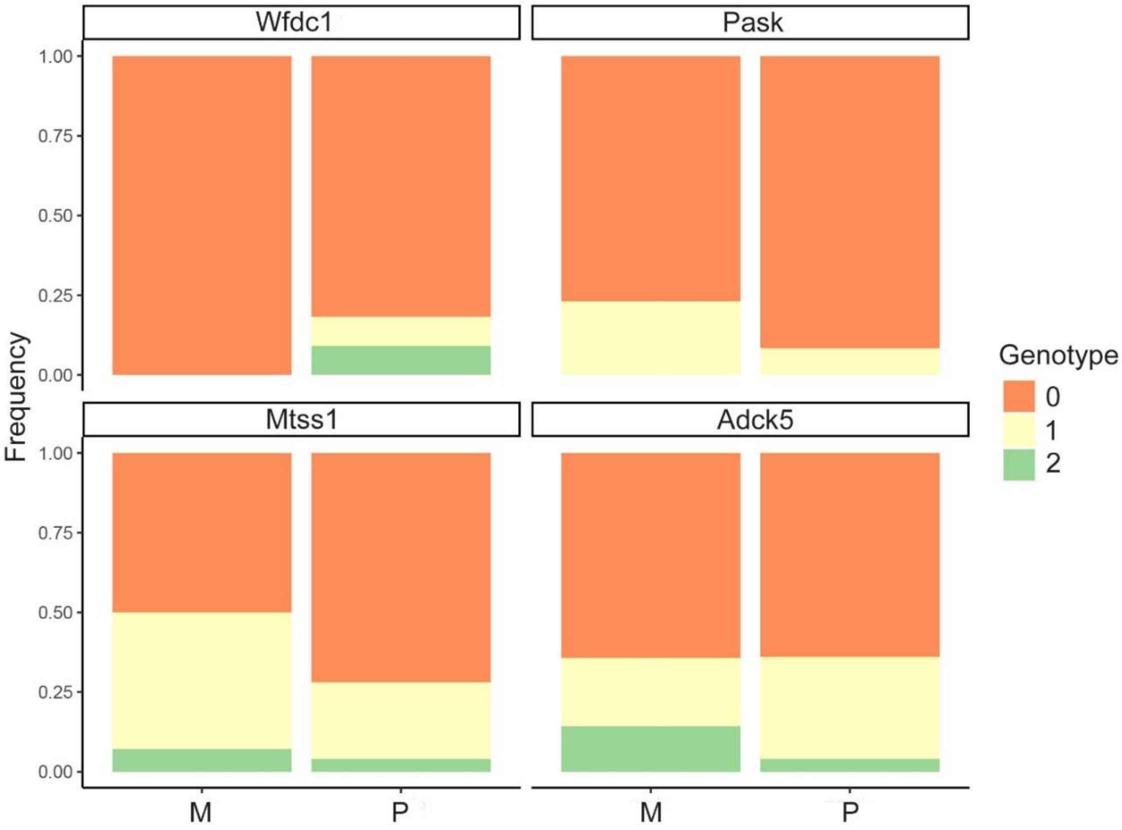

Figure 4.2: Genotype frequencies of the four QTLs for each female morph (M: metamorphic and P: paedomorphic). 0=homozygous at the reference allele, 1=heterozygous, 2=homozygous at the alternative allele.

Εικόνα 4.2: Γονοτυπικές συχνότητες των τεσσάρων πιθανών γονιδίων για κάθε μορφή θηλυκού (M: μεταμορφωμένο και P: παιδομορφικό). 0=ομόζυγο για το αλληλόμορφο αναφοράς, 1=ετερόζυγο, 2= ομόζυγο για το εναλλακτικό αλληλόμορφο.

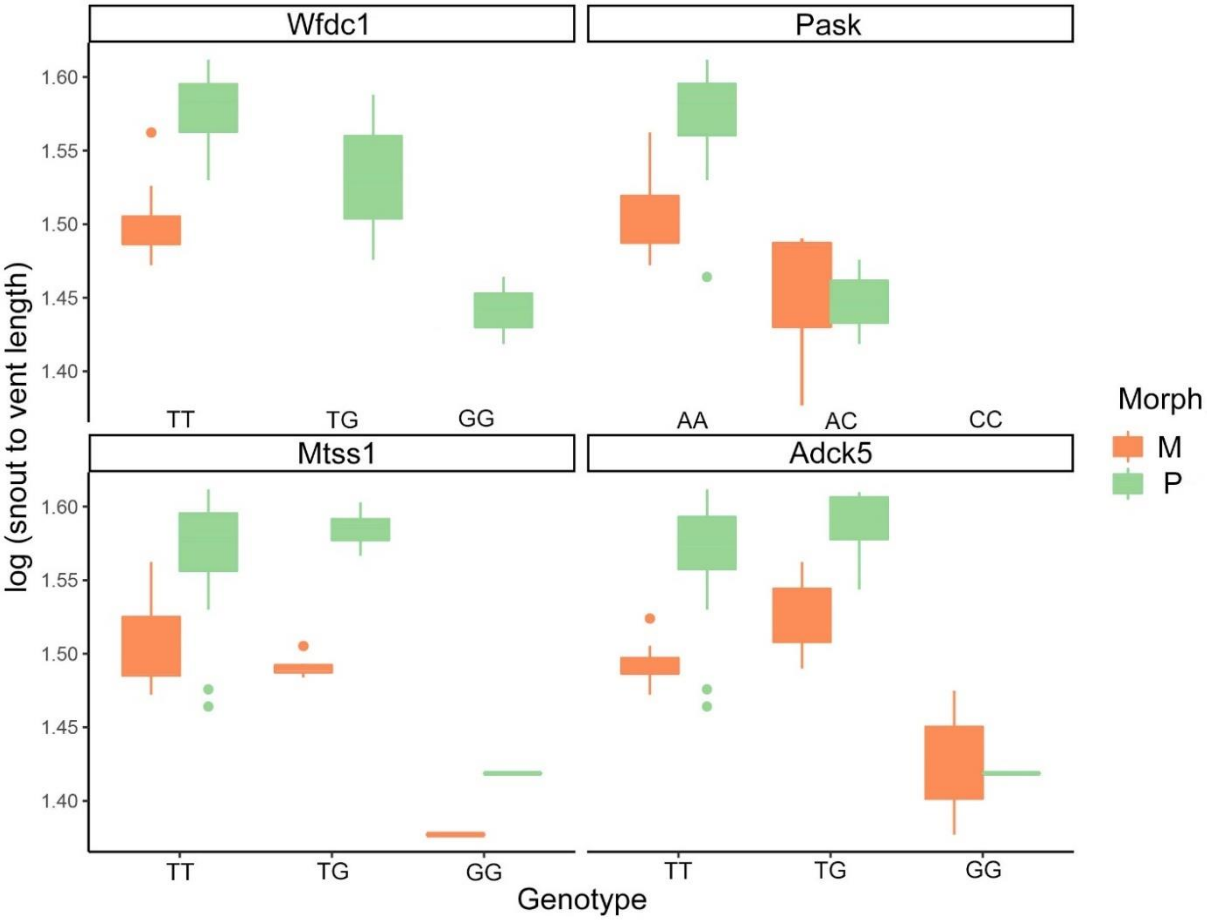

Figure 4.3: Mean body size of the Greek smooth newt in females of both morphotypes for each of the putatively associated loci on the identified genes. Whiskers represent 95% of Confidence Intervals.

Εικόνα 4.3: Σωματικό μέγεθος των θηλυκών τριτώνων για κάθε μορφότυπο στους τέσσερις πιθανούς πολυμορφισμούς στα αναγνωρισμένα γονίδια. Παρουσιάζονται και τα 95% διαστήματα εμπιστοσύνης.

4.4 Discussion

In the present study, I utilized a reduced representation sequencing method to obtain thousands of SNPs for a wild population of the Greek smooth newt (*Lissotriton graecus*) exhibiting facultative paedomorphosis. By performing a genome-wide association study, I was able to identify loci strongly associated with the phenotypic trait of body size and identify candidate genes putatively associated with this trait.

Here, four candidate genes highly correlated with the phenotypic trait of body size, were identified, although the lack of a reference genome in my case may prevent a more in-depth interpretation of the results. The *Adck* family proteins are encoded by several genes that have protein serine/threonine kinase activity, including the identified here *Adck5* which is suggested to have a similar function with the *Adck1* gene (Yoon et al. 2019), that is regulating mitochondrial fusion and the formation of cristae. *Pask* is an evolutionary conserved gene that may be involved in energy homeostasis by regulating glycogen synthesis and have a pivotal role in respiratory regulation (DeMille & Grose 2013). The *Mtss1* gene is predicted to be involved in negative regulation of cell proliferation. It encodes an actin-binding protein that plays a key role in cell adhesion, movement and growth, while studies show the importance of this gene in the developing of the nervous system and tissue differentiation (Glassmann et al. 2007, Sistig et al. 2017). Finally, the *Wfdc1* gene encodes a protein highly conserved in vertebrates which has been implicated in tissue homeostasis and wound repair (Ressler & Rowley 2011, Solís-Calero & Carvalho 2019). It is suggested to have a pivotal role in cell proliferation and adhesion, while recent studies show an involvement in immune responses (Ressler et al. 2014).

The identified GO terms in the present study confirm the role of the identified candidate genes in epithelial cell proliferation, while serine/threonine kinases are a class of enzymes that phosphorylate proteins, an important modification central to many biological processes like cell proliferation and regulation of cell apoptosis (Brady 2005). Cell proliferation, the increase in the number of cells, is an important factor from embryogenesis to tissue repair and growth and is regulated by complex mechanisms. Epithelial cells form a tightly packed tissue that cover organs, blood vessels, internal cavities and, also form the skin. During amphibian development and metamorphosis, several epithelial cells undergo rapid proliferation, while growth and body size is dependent on the subsequent differentiation of these cells (Ishizuya-Oka 2007, Puliafito et al. 2017).

Regarding levels of heterozygosity, I did not observe any correlation of fitness-related traits with genome-wide heterozygosity, nor the heterozygosity calculated from the trait-associated loci. Few studies have confirmed the presence of heterozygosity-fitness correlations hence it is difficult to evaluate the generality of such relationships in wild populations (Szulkin et al. 2010). Although my dataset contained thousands of SNPs, several studies show the varying results of HFCs regarding the number and type of loci used (Bateson et al. 2016, Gagnon et al. 2019). This highlight the need of careful consideration of the type of markers and what filtering procedures should be taken into consideration when assessing heterozygosity in the newts' large and complex genomes.

Notably, although allele differences of the variants were not observed between the two morphotypes in female newts, there were differences among the genotypes. Carriers and homozygous individuals of the alternative allele on the putatively associated variants exhibited smaller body size than homozygous on the reference allele in both morphs, while interestingly, metamorphic female newts exhibited higher genotype frequencies of homozygosity in the *Adck5* which led to the smaller observed body size. Even though the identified variants in the study have been identified to specific genes, whether these variants have a direct effect on the phenotypic trait or are in vicinity of true functional SNP is unclear and cautious should be taken in interpreting the results.

In the present study, I performed a genome-wide association study which revealed marker loci highly associated with the trait of body size in the Greek smooth newt. Those variants were successfully annotated to genes that have a crucial role in cell proliferation, however the specific role of those variants is unclear. These results offer the possibility for further examination of genetic variation at the specific loci and inspection of the underlying genetic models acting on functional genes. Future studies should incorporate more traits associated with body size, such as age and secondary sexual traits, to disentangle the genetic basis of the complex quantitative trait of body size.

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Chapter 5

Persistence and extinction risk of a Greek smooth newt population, exhibiting facultative paedomorphosis

Κεφάλαιο 5

Διατήρηση και κίνδυνος εξαφάνισης ενός πληθυσμού του ελληνικού κοινού τρίτωνα που εμφανίζει περιστασιακή παιδομόρφωση

5.1 Introduction

Viability of species is often described by the extinction risks and/or recovery rates of populations over a period of time (Akçakaya & Sjögren-Gulve 2000). Population Viability Analyses (PVA) predict population trajectories and persistence by incorporating stochastic and deterministic factors (Brook et al. 2000) and have been a valuable tool in conservation biology and species protection.

Amphibians are one the most threatened vertebrates and there have been concerns about their population declines worldwide. Specifically, 47% of salamander and newt populations are listed as Endangered (Stuart 2008). Environmental drivers like pond desiccation, anthropogenic pressures such as pollution and habitat fragmentation, introduction of invasive species and spread of pathogens have been proposed to be behind the observed amphibian declines (Walther et al. 2002, Beebee & Griffiths 2005, Stuart 2008). Newts have a valuable role in freshwater ecosystems, acting as indicators of environmental stress (Blaustein & Wake 1990). Since they depend on both aquatic and terrestrial habitats, they are prone to habitat fragmentation and alterations such as acidification, eutrophication, draining and fish introduction (Denoël et al. 2009).

Especially, newts that exhibit facultative paedomorphosis, the retention of larval characteristics such as gills in sexually mature individuals, have been facing the detrimental effects of pond desiccation and introduction of alien fish species (Denoël et al. 2005, Mathiron et al. 2017). Facultative paedomorphosis is an example of phenotypic plasticity which grants to individual the ability to adapt in changing environments and escape poor aquatic conditions by metamorphosing to the terrestrial morph (Mathiron et al. 2017, Toli et al. 2020). However, this phenotypic plasticity is characterized as “fixed” since it can happen only once during the lifespan of the individual, leading to potentially decrease of fitness when also the terrestrial habitat becomes hostile (Chevin et al. 2013). Phenotypic diversity has been long recognized to be fundamental for the ecological success of populations and evolution (Forsman 2014).

Although, several cases of extirpation and loss of the paedomorphic morphotype have been observed in newt populations due to introduction of invasive fish species, pond desiccation and habitat fragmentation, the resilience of the phenotype is possible after the removal of the environmental stressor through conservation actions (Denoël & Winandy 2015, Denoël et al. 2019).

PVAs have been widely used to predict the extinction risk of newt populations and evaluate how management actions, like habitat restorations, supplementations and improve of landscape connectivity, would impact their preservation (Halley et al. 1996, Edgar et al. 2005, Karlsson et al. 2007).

Here I focus on a Greek smooth newt (*Lissotriton graecus*) population that exhibits high rates of facultative paedomorphosis (Sotiropoulos et al. 2017). Although, *Lissotriton vulgaris* complex is considered as Least Concern according to the IUCN, population declines have been observed for all the species included in *L. vulgaris sensu lato* (Denoël 2012). The species occupies both natural and artificial ponds surrounded by pastureland and deciduous/mixed woodland while pond macrophyte cover and connectivity have been identified as the most important parameters for predicting occurrence (Bounas et al. 2020). During an experiment I collected data on fecundity of female smooth newts and survival rates from the hatching till the sub-adult stage of larvae (see Chapter 2), along with ratios of metamorphosis of adult female and male newts due to fish introductions (Toli et al. 2020). By utilizing these life history data along with existing knowledge from the literature along with genetic data (see Chapter 3), I aim to 1) predict the extinction risk of the studied paedomorphic population, 2) investigate the sensitivity of the paedomorphic phenotype to pond desiccation and fish introductions, 3) explore possible sex-specific differences in the persistence of the alternative morphotype, and lastly 4) examine the effects of each scenario to the levels of gene diversity.

5.2 Methodology

5.2.1 Study site

The site used in the study contains an artificial fishless pond where a Greek smooth newt population, exhibiting high levels of facultative paedomorphosis, breeds (Sotiropoulos et al. 2017). The surrounding habitat is characterized mostly by abandoned cultivations and extended pastures of herbaceous vegetation, with sparse deciduous trees. The pond has not been used for livestock or cultivation watering for the last 15 years and is used as a breeding site for several amphibian species, such as *Bufo bufo*, *Hyla arborea*, *Pelophylax epeiroticus*, *Bufo viridis* and *Triturus macedonicus*. The population is isolated with the nearest known breeding site of other Greek smooth newt populations being 3.55 km away.

5.2.2 PVA modelling and parameters

Population Viability Analyses were performed using VORTEX v. 10.4.1, which allows the input of demographic and genetic data to determine the risk of extinction of the focal population (Lacy 1993). Each scenario was run with 10,000 iterations over a 25-year period (Table 5.1). Shorter time frames were used since long term analyses are prone to errors and uncertainty (Beissinger & Westphal 1998). A stage-structured model was used (eggs, larvae, juveniles, and adults). Newts follow a polygynous mating system where males may mate with several females (Jehle et al. 2007), hence the number of males may not affect fecundity (Akcakaya 2000).

Age, reproduction, and survival

Smooth newts breed for the first time at 2 or 3 years old, and then every year, while longevity has been estimated from 5 to 10 years (Bell 1977, Cogalniceanu & Miaud 2003). Populations from permanent habitat mature at younger age while longevity increases in contrary to populations from unpredicted environments (Nobili & Accordi 1997). I assumed an age at maturity of 2 years old for both sexes, and the upper age limit was set to 10 years. Reproduction parameters were based on a fitness experiment (see Chapter 2) and the literature. Briefly, egg production and larval development produced by female Greek smooth newts were recorded from 61 individuals. Females were placed alone in aquatic tanks, where the number of deposited eggs, number of hatched eggs and number of survived larvae till metamorphosis were recorded. Fecundity was derived from egg mortality and larval survival and calculated as the average number of individuals produced by each female. The annual survival rate of adult newts was set to 55% and 45% for females and males, respectively (Bell 1977), while juvenile survival was set to 80% (Bell 1977).

Density dependence

Density dependence processes have a crucial role in population dynamics and long-term viability, by affecting several aspects like survivorship, growth rates, fecundity and dispersal in an amphibian population, although the exact effects in newt populations are poorly known (Vignoli et al. 2018, Cayuela et al. 2019). Small populations exhibit strong demographic and stochastic effects (Lacy 2000), while low densities increase the difficulty in finding mates (Cayuela et al. 2019). Hence, the model tested a quasi-extinction rather than total extinction; total number of individuals remained was set to 10, and a ceiling model was incorporated to affect vital rates. High density decreases growth rates and survivorship in larvae and adults, through competition (Petranka & Sih 1986, Vignoli et al. 2018). Lastly, I assumed that 100% of males would be present in the breeding pool.

Initial population size and carrying capacity

Population size was estimated by a capture-recapture method, where newts were captured using dip-nets in monthly samplings (Moustakas et al. 2018). Newts were classified by sex and stage and their belly patterns were photographed. Individuals showing developed cloaca and secondary characteristics such as toe webs and tail filaments, were classified as adults (Denoël 2016). Using the Wild-ID software (Bolger et al. 2011) along with the program Mark (McClintock & White 2012), the demographic parameters of the Greek smooth newt population were estimated under the Jolly-Seber model (Jolly 1965, Seber 1965). The initial population size N was based on the mean monthly estimates of the capture-recapture (Mantzana-Oikonomaki et al. 2018), while the carrying capacity was set at 100% higher than the initial population size with a 20% increase due to underestimation. During the monthly samplings at the study site, the observed sex ratios were also recorded (Toli et al. 2017).

Genetic Input

To investigate the effects of each scenario to the genetic variation of the population, allele frequencies of 27,274 SNPs were genotyped in a total of 64 newts, using a customized dd-RAD protocol (see Chapter 3). Due to the large amount of data, I analyzed three subsets of 1,000 random SNPs, hence the mean values of gene diversity were retrieved.

5.2.3 Scenario projections

The scenarios investigated, that considered to explicitly affect the persistence and expression of facultative paedomorphosis, were the introduction of invasive fish species and pond desiccation. VORTEX can model environmental stochasticity by incorporating catastrophes, which are an extreme example of environmental variation and have an important influence in the reproduction and survival of individuals (Akcakaya 1998). Fish introduction was modeled to increase metamorphosis ratio by 75% in female and 100% in male Greek smooth newt according to a study on the effects of *Gambusia hoolbrooki*, a highly invasive fish species, on paedomorphic individuals of the same study population (Toli et al. 2020), while female reproduction was decreased by 40% as estimated in the same study. Pond desiccation is a common factor that causes high rates of metamorphosis in paedomorphic newts, while studies show a 40% metamorphosis ratio in females and 80% in males (Mathiron et al. 2017). Although drought and fish introduction cause high mortality rates in eggs and larvae of pond-breeding amphibians (Denoël 2003, Cabrera-Guzmán et al. 2017), in this case I did not include an effect in reproduction of adult females.

Table 5.1: VORTEX parameter inputs for the basic model and scenario projections.

Πίνακας 5.1: Παράμετροι που χρησιμοποιήθηκαν στην ανάλυση VORTEX για το αρχικό μοντέλο και τα σενάρια.

Parameter	**Value**
Extinction definition	Quasi-extinction, 10 individuals
Species description	
Inbreeding depression	No
EV Correlation between reproduction and survival	0.5
Reproductive system	Polygynous
Age of 1st offspring females/males	2
Maximum lifespan (years)	10
Maximum age at Reproduction (years)	10
Density dependence	Ceiling model at carrying capacity

Reproductive rates	
% adult females breeding	80%
EV (SD) in % Breeding	20%
Mortality rates	
From egg to hatch	0.39±0.3
From hatched to juvenile	0.41±0.3
From juvenile to adult	0.20±0.1
Mortality of female adults	0.45±0.1
Mortality of male adults	0.55±0.1
Mate monopolization	
% males in breeding pool	100%
Initial population size (nr of individuals)	1,424
Carrying capacity K (nr of individuals)	2,484±20% SD

5.3 Results

Population size estimates obtained with capture-recapture method is shown in Figure 5.1, while the study population showed a female-biased sex ratio throughout the sampling sessions (Figure 5.2). The paedomorphic population is declining but has a probability of persistence over a 25-year period of 35.4%, while the deterministic growth rates of the Greek smooth newt population at all cases was negative. Both fish introduction and pond desiccation are predicted to have a significant impact on the paedomorphic population by reducing the time to the first population extinction (Table 5.2). Fish introduction would lead to total extinction of the paedomorphic morphotype in both sexes, while pond desiccation would have a significant impact in male paedomorphic newts (Figure 5.3). Loss of gene diversity is observed with pond desiccation and introduction of invasive fish (Table 5.2).

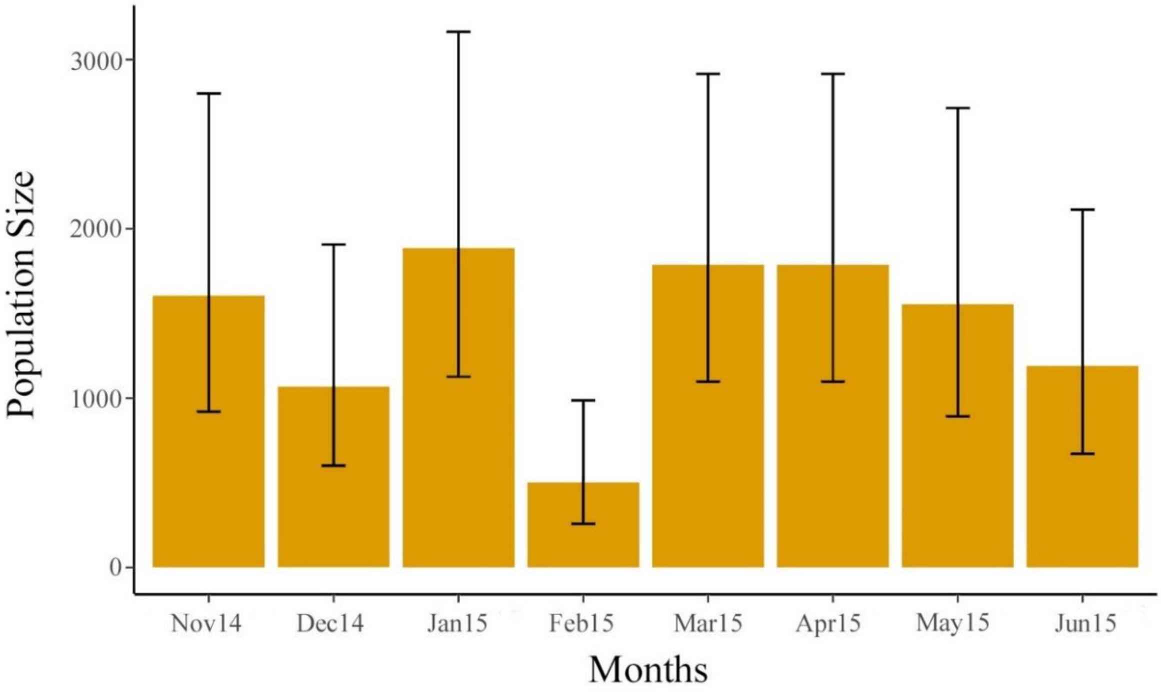

Figure 5.1: Monthly population estimates of the Greek smooth newt population based on the capture-recapture method. Confidence Intervals 95% are shown (Mantzana-Oikonomaki et al. 2018).

Εικόνα 5.1: Μηνιαίες εκτιμήσεις του μεγέθους πληθυσμού σύμφωνα με τη μέθοδο της σύλληψης-επανασύλληψης με 95% όρια εμπιστοσύνης (Mantzana-Oikonomaki et al. 2018).

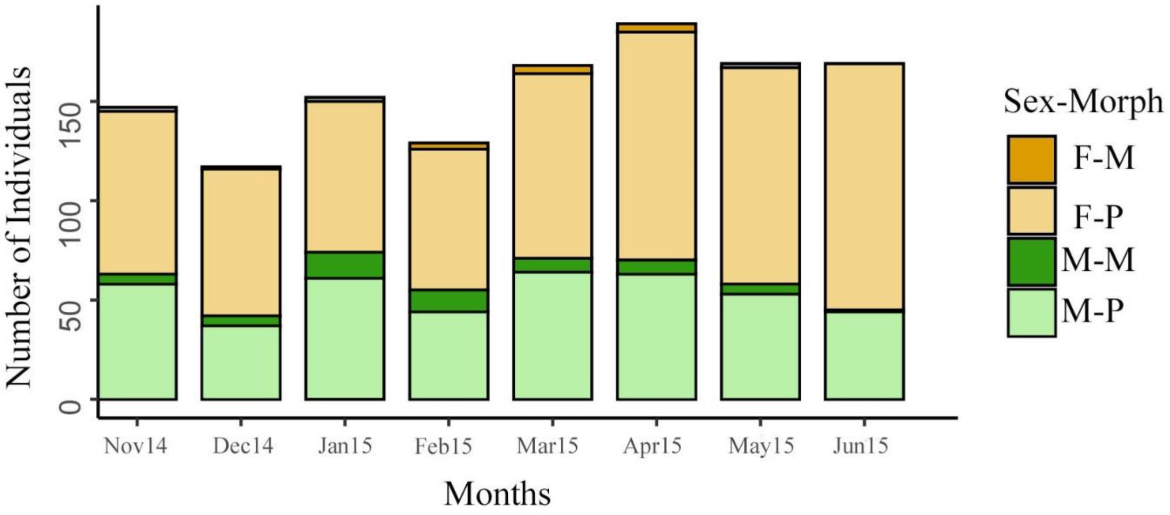

Figure 5.2: Number of newts caught in each sampling session according to sex (F: female and M: male) and morph (M: metamorph and P: paedomorph).

Εικόνα 5.2: Αριθμός ατόμων ανάλογα με το φύλλο (F: θηλυκό και M: αρσενικό) και τη μορφή (M: μεταμορφωμένο και P: παιδομορφικό), που βρέθηκαν σε κάθε δειγματοληψία.

Table 5.2: Population Viability Analyses outputs from simulations of fish introductions and pond desiccation for each sex in the studied population of *Lissotriton graecus*. stoch-r: stochastic growth rate with SD values; PE: the probability of population extinction after 25 years; N: mean population size with SD values; GD: gene diversity (expected heterozygosity) with SD values. TE: median time in years to first population extinction.

Πίνακας 5.2: Αποτελέσματα της ανάλυσης βιωσιμότητας για κάθε σενάριο στον πληθυσμό του *Lissotriton graecus*. stoch-r: στοχαστικός ρυθμός ανάπτυξης ±SD; PE: πιθανότητα εξαφάνισης μετά από 25 χρόνια; N: μέσος αριθμός πληθυσμού ±SD; GD: γενετική ποικιλότητα (παρατηρούμενη ετεροζυγωτία) ±SD. TE: μέσος αριθμός χρόνων ως την πρώτη εξαφάνιση.

Scenarios		stoch-r	SD(r)	PE	N	SD(N)	GD	SD(GD)	TE
0	Base model	-0.148	0.492	0.354	104.44	234.23	0.227	0.029	0
1	Fish introduction - Females	-0.759	0.882	1	0	0.03	0.00	0.00	6
2	Fish introduction - Males	-0.171	0.481	1	0	0	0.00	0.00	2
3	Pond desiccation - Females	-0.286	0.537	0.931	3.27	22.43	0.188	0.03	15
4	Pond desiccation - Males	-0.551	0.847	0.999	0.02	0.48	0.188	0.03	8

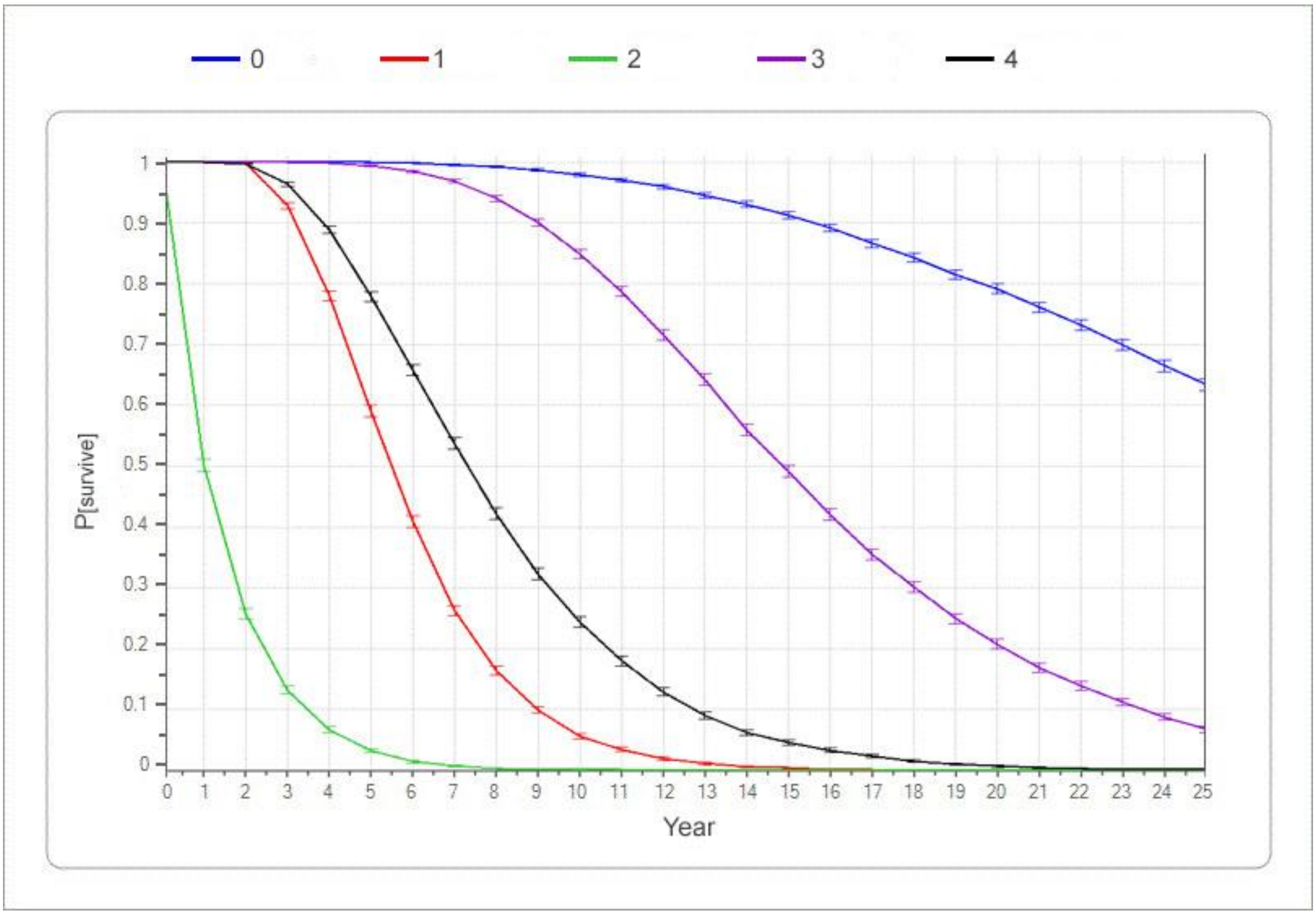

Figure 5.3: Probability of survival of the paedomorphic Greek smooth newt population under the base model (0) and the different scenarios (1-4) (see Table 5.2) over a 25-year period. 95% Confidence Intervals are shown.

Εικόνα 5.3: Πιθανότητα επιβίωσης του παιδομορφικού πληθυσμού του ελληνικού κοινού τρίτωνα σε κάθε σενάριο (1-4) (Πίνακας 5.2) για διάστημα 25 χρόνων. Παρουσιάζονται τα 95% όρια εμπιστοσύνης.

5.4 Discussion

My study shows the sensitivity of the local paedomorphic population to fish introduction and pond desiccation, along with sex-specific differences in the persistence of the paedomorphic morphotype. The base model shows a negative growth rate but a persistence of the paedomorphic population within the next 25 years. However, the probability of the paedomorphic morphotype to become extinct is high in the face of the environmental stressors.

Fish introductions and pond desiccation are two of the most common stressors of amphibian populations, since many invasive fish species are relocated to ponds and wetlands for recreational fishing, while climate change leads to higher frequencies of drought. There have been several reports of amphibian population declines and, even extinction, after the introduction of invasive species acting as predators (Denoël et al. 2005, Denoël & Lehmann 2006, Winandy et al. 2015). Newt populations are facing the detrimental effects of these predators by minimizing reproductive behaviors and avoiding the hostile aquatic environment by metamorphosing, leading to the extirpation of the paedomorphic phenotype (Winandy & Denoël 2013, Winandy & Denoël 2015, Toli et al. 2020). Nonetheless, management actions including fish removal and habitat restoration can prevent further population declines and lead to the recurrence of the paedomorphic phenotype (Winandy & Denoël 2015, Miró et al. 2020).

On the other hand, small wetlands, that are critical to freshwater biodiversity and have important role in ecosystems, have been recognized to be vulnerable and sensitive both to anthropogenic pressure and climate change, which leads to increasing frequency of droughts (Biggs et al. 2017, Préau et al. 2020). Amphibian populations depend on small waterbodies like lakes, ponds, and streams, while at the same time populations that exhibit paedomorphosis spent most of their life span in the aquatic habitat (Whiteman 1994, Denoël et al. 2005). Facultative paedomorphic individuals can metamorphose later in their life span to avoid the stressful conditions due to desiccation, however the extended periods of drought can lead to the extinction of the paedomorphic phenotype. Although, there have been reports of paedomorphic newts migrating to nearby locations without metamorphosing, hence preserving the alternative morphotype (Denoël 2003), in this case the population is isolated without any alternative aquatic habitats present in proximity.

Sex-specific differences in the persistence of the paedomorphic morphotype are evident in my study, since paedomorphic males have the higher probability of extinction in the presence of both environmental stressors. Higher rates of metamorphosis in males have been observed in studies of the palmate newt, *Lissotriton helveticus*, while sex ratios of wild paedomorphic populations have been observed to be female-biased supporting the "male escape hypothesis" (Whiteman 1997, Mathiron et al. 2017). Male newts metamorphose earlier than females in stressful environmental conditions, probable due to differences in life history traits since females may benefit from longer stay in the aquatic habitat to fuel their energy sources for breeding (Denoël et al. 2019). My models corroborate the importance of sex in the persistence of paedomorphosis in the Greek smooth newt and its significance in population viability models.

Loss of genetic variation may lead to loss of potentially adaptive variation, compromising the persistence and viability of populations (Amos & Balmford 2001). Here, both scenarios lead to decrease of genetic diversity, however caution should be taken into consideration since environmental stressors lead to extirpation of the phenotype and not the population itself. Newts will metamorphose to avoid the hostile habitat and reports have confirmed the detrimental effects of fish and pond desiccation to the reproduction and viability of newly hatched larvae, however the metamorphic newt population may remain at the site or migrate and colonize nearby aquatic sites (Winandy et al. 2015, Cabrera-Guzmán et al. 2017, Davenport et al. 2017).

Although, population viability analyses offer insight in the persistence of population and may predict its future course, caution should be taken since several key assumptions in the models may be highly population specific and could bias estimates of extinction risks. Here, juvenile and adult survival rates were assumed according to literature (Bell 1977), however these estimates may be highly specific and differ among populations. Reproductive and survival estimates were based only on two years collected data, so longer study of annual reproductive output could help refining the analysis. Furthermore, according to Mantzana-Oikonomaki et al. (2018), the success in recognition and identification of recaptured individuals is highly affected by the intense color patterns, an attribute lacking in paedomorphic Greek smooth newts. The lower recapture and identification rates led to estimates with higher uncertainty, hence, further information on population size estimates could improve the model.

Further study of life history data on other newt population exhibiting facultative paedomorphosis may increase the accuracy of future analyses. Incorporating genetic data and reports on fecundity, along with studies on the effects of environmental stressors on metamorphic newts and larval survival, will result in higher resolution of viability studies in local newt populations. Future work should focus on possible management actions for the preservation of paedomorphosis, an important alternative ontogenetic trajectory, and the development of models that have central part in conservation planning.

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Chapter 6

Consequences of ecological interaction with alien species in the expression and conservation of alternative phenotypes

Κεφάλαιο 6

Επιπτώσεις των οικολογικών αλληλεπιδράσεων με ξενικά είδη στην έκφραση και διατήρηση εναλλακτικών φαινοτύπων

Based on:
Toli, E. A., Chavas, C., Denoël, M., Bounas, A., & Sotiropoulos, K. (2020). A subtle threat: behavioral and phenotypic consequences of invasive mosquitofish on a native paedomorphic newt. *Biological Invasions*, *22*(4), 1299-1308.

6.1 Introduction

Worldwide introductions of alien species significantly alter local ecosystems and have great impact on the native species, especially on freshwater communities, that are considered to be extremely vulnerable (Bucciarelli et al. 2014, Strayer 2010). Alien fish consist an additional threat to the local amphibian populations, which are already under pressure due to climate change (Walther et al. 2002), human activities (Blaustein & Kiesecker 2002, Beebee & Griffiths 2005, Denoël 2012) and the emergence and spread of pathogens and diseases (Garner et al. 2009, Raffel et al. 2010). Fish invaders can prey upon larvae, eggs, and sometimes even adults of native amphibians, compete with them for important resources such as food and shelter, and alter the chemical and structural characteristics of aquatic habitats making them hostile for many species (Orizaola & Brana 2006, Hartel et al. 2007, Joseph et al. 2011).

Since the early twentieth century, fish species, like guppies and mosquitofish, were widely introduced and have been established in several freshwater ecosystems worldwide, as a means of malaria control (Vidal et al. 2010, El-Sabaawi et al. 2016). One of the most successfully introduced species is the eastern mosquito fish (*Gambusia holbrooki*), which utilizes a wide range of habitats and shows high ecological tolerance (Pyke 2005), making it a successful invader. It’s a small, live-bearing fish native to North America, and feeds primarily on zooplankton and small vertebrates (Crivelli & Boy 1987). The species was originally introduced for the biological control of mosquitoes in Spain in 1920s and has expanded since then in many freshwater ecosystems throughout Europe (Pyke 2008). However, several studies showed that this action has not worked as expected since mosquitofish were found to prey on different invertebrates than mosquito larvae (Garcia-Berthou 1999, Remon et al. 2016), resulting in pervasive impacts to native wetland communities (Shulse et al. 2013). The ecological impacts of this invasive species have been the focus of several studies that aimed to study the direct and indirect effects on native species, especially on amphibian populations. Mosquito fish prey upon amphibian larvae and tadpoles, decrease abundance and larvae development and interfere with vital activities like breeding (Cruz et al. 2006, Cabrera-Guzmán et al. 2018, Vannini et al. 2018).

Many amphibians exhibit complex life cycles, utilizing both aquatic and terrestrial habitats during their life span (Wilbur 1980). However, some salamander and newt species follow an alternative developmental pathway known as paedomorphosis, where adult individuals reach adulthood without metamorphosing (Wilbur 1980, Whiteman 1994, Denoël et al. 2005). In facultative paedomorphic species, paedomorphs retain the possibility to metamorphose, for example due to environmental drivers such as pond drying (Mathiron et al. 2017). Since, paedomorphic individuals spend their entire (or most) life span in the aquatic habitat, they are more prone to alterations of the aquatic habitat and have been recognized to be extremely vulnerable to fish introductions (Denoël & Winandy 2015, Denoël et al. 2019a).

The eastern mosquito fish was introduced in Lake Pamvotis in Epirus in the mid of 1950s and now occurs throughout several aquatic habitats in Ioannina basin (Gkenas et al. 2012). At the same time, the region of Epirus sustains paedomorphic populations and recently, cases of paedomorphic populations of *Triturus macedonicus* have been reported, increasing the importance of this area (Denoël 2004, Sotiropoulos et al. 2008, Sotiropoulos et al. 2017). Since mosquitofish show high population growth rates and dispersal tendencies (Cote et al. 2010), they raise concerns for the negative impacts to the native amphibian populations, including those exhibiting paedomorphosis.

To this end, I aim to investigate and quantify the impact of the most invasive fish species on paedomorphic newts as a result of direct physical disturbance and/or indirect modification of their behavior, and the effects on metamorphosis rate as well.

6.2 Methodology

6.2.1 Experimental Design

During the breeding season (April 2017), 10 male (SVL; i.e. the length from the tip of the snout to the end of cloaca: mean SVL ± SE: 33.6 ± 0.6 mm) and 10 female (35.6 ± 1.2 mm) paedomorphic Greek smooth newts, i.e. adult gilled individuals, were caught by dip-nets from the studied site in the University of Ioannina Campus. All individuals were brought directly to the laboratory in large containers filled with pond water and were placed randomly in pairs in aquatic tanks. Additionally, 10 male (SL; from the tip of the snout to the posterior end of the last vertebra: mean SL ± SE: 20.5 ± 1.5 mm) and 20 female (36 ± 1.5 mm) mosquitofish were collected from another natural pond in Ioannina basin (39°34’N 20°52’E, 481 m a.s.l).

We separated tanks in two treatments: five tanks without fish (control) and five with fish. We placed one male and two female mosquitofish in each tank of the respective treatment. So, in total, there were 5 tanks with a pair of newts (1 male and 1 female) and 5 tanks with a pair of newts along with 1 male and 2 female mosquitofish. Newts and fish were fed ad libitum with black mosquito larvae (*Culex sp.*) and bloodworms (*Chironomus sp.*) every afternoon.

Aquatic tanks (38x9x24 cm, 22 cm water depth; 20 L) were filled with dechlorinated tap water, the bottom was floored with gravel, and kept at a temperature of 16°C and 13h light–11h dark photoperiod. All aquatic tanks were provided a plastic floating platform (8.5x9x8.5 cm), a cylindrical plastic shelter attached to the bottom of the tank (length 10 cm, diameter 4 cm) and strips of plastic that were fixed on the bottom of each tank to simulate aquatic vegetation (Figure 6.1).

Figure 6.1: Aquatic tank used in the experiment, where a) floating platform, b) shelter, c) plastic strips and d) gravel.

Εικόνα 6.1: Ενυδρείο που χρησιμοποιήθηκε στο πείραμα όπου φαίνονται α) η επιπλέουσα επιφάνεια, β) το κυλινδρικό καταφύγιο, γ) οι πλαστικές λωρίδες που προσομοιάζουν υδρόβια βλάστηση και δ) το υπόστρωμα.

The tanks were distributed randomly in the lab to avoid biases, and visual contact of neighboring tanks was blocked using thick paper. The water was renewed every 5 days using dechlorinated tap water from tanks stored at the experimental temperature. The experiment lasted four weeks. After the end of the experiment, newts were released back to their habitat, while mosquitofish were euthanized with an overdose of MS-222 following the guidelines for handling species in scientific research according to the European Directive 2010/63/EU.

6.2.2 Video Analysis

Each aquatic tank was video recorded four times a day for 10 min duration for each video (video recorders TurboX Act-150 were set at a resolution of 1280 * 720 pixels and 30 frames per second) over the 4-week period. We recorded two times in the morning and two in the afternoon with an interval of two hours. Video analysis was done using the BORIS software for behavioral analysis (Friard & Gamba 2016) and an ethogram was produced where five behaviors of paedomorphic newts were quantified:

a) Moving in open areas (i.e. swimming in the water column, moving on gravel, air taking),

b) Immobility in open areas (i.e. no visible movement, motionless),

c) Hiding when newts were using the shelter,

d) Oviposition when females laid eggs, and
e) Courtship when males engaged in sexual display.
In addition, I scored fish attacks to newts and noted the recipient of the attack (i.e. either the male or the female newt).

In order to assess the foraging behavior of newts, I tested the feeding activity five times during the 4-week experiment, which involved the foraging on an earthworm (*Lumbricus terrestris*, approx. 5–6 cm long). The earthworm was attached to a string and immersed in each aquarium so it would be visible by all newts. I then set a video recording for 10 min in order to assess the feeding activity of newts:

a) Engagement in a feeding act (feeding vs. not feeding) and
b) The latency by measuring the time from food delivery to the first feeding act by a newt (the minimum foraging latency)

Lastly, to assess the rate and timing of metamorphosis, I visually inspected newts daily for signs of metamorphosis (closed gill slits), which coincides with a transition to the terrestrial habitat (Mathiron et al. 2017).

6.2.3 Statistical Analysis

The statistical analysis considered only behaviors that were done at the paedomorphic stage. Post metamorphic data were discarded. The data were analyzed using linear mixed models (LMM) and generalized linear mixed models (GLMM) as implemented in package ‘‘lme4’’ (Bates et al. 2014) in R v. 3.4.1. (R Core Team 2019). I fitted a LMM on duration for moving and immobility, using treatment (control vs. fish), sex and their interaction as fixed effects and the identity of individuals and tanks as a random effect. A GLMM was performed on hiding data under a binomial distribution (hiding vs. not hiding), using treatment (control vs. fish), sex and their interaction as fixed effects and the identity of individuals and tanks as a random effect. Due to the rareness of events, the analysis for oviposition (number of eggs laid per female) was carried out using summarized data of the 4-week period for each tank. Scores corresponded to counts (i.e. number of eggs laid) out of each 10 min sequence. I then fitted a GLMM for count data (Poisson distribution), using treatment as fixed factor and the identity of tank as a random effect. For courtship behavior of male newts, I did not perform any statistical analysis, since only two males in the control and only one male in the fish treatment were engaged in sexual displays, thus preventing any statistical comparisons.

To assess the feeding activity of newts, a GLMM was fitted with binomial distribution (feeding vs. not feeding) using treatment, sex and their interaction as fixed effects and individuals as a random effect. For minimum foraging latency I fitted a survival analysis for right-censored data, as implemented in the ‘‘Survival’’ R package (Therneau & Lumley 2015) and ordinary log rank tests were performed to test for differences between the groups. Post hoc comparisons for significant interactions were implemented in ‘‘lsmeans’’ package (Lenth 2016).

In order to investigate the effect of treatment and sex on the rate and timing of metamorphosis during the 4-week period, a survival analysis for right-censored data was performed using a non-parametric method, the Kaplan–Meier estimator, as implemented in the ‘‘Survival’’ R package. Ordinary log rank tests were performed to test for differences of the survival curves between the groups, using the Bonferroni adjustment to correct for multiple comparisons.

6.3 Results

At the end of the experiment, I obtained 104.3 hours of recorded videos for the ten tanks. The average ± SE number of videos per individual was 57.8 ± 2 (range 31–64 videos).

Summarized data and results on each recorded behavior are shown in Table 6.1 and 6.2. A significant main effect of treatment (LMM: $F_{1,10} = 7.533$, $p = 0.02$) and sex (LMM: $F_{1,10} = 5.714$, $p = 0.037$) was found on moving. No statistically significant effects of treatment, sex and their interaction were found on immobility.

Table 6.1: Oviposition (total number of eggs laid) and proportion of time (mean±SE) spent for each recorded behavior during the 4-week experiment for female and male paedomorphic newts in control and fish treatment.

Πίνακας 6.1: Ωοαπόθεση (συνολικός αριθμός αυγών) και ποσοστό του χρόνου (mean±SE) που αφιερώθηκε σε κάθε συμπεριφορά κατά τη διάρκεια του πειράματος 4 εβδομάδων για τα θηλυκά και αρσενικά παιδομορφικά άτομα στα ενυδρεία ελέγχου και στα ενυδρεία παρουσία ψαριών.

Treatment	**Sex**	**Oviposition**	**Moving**	**Immobility**	**Hiding**	**Courtship**
Control	Female	28	0.509±0.018	0.353±0.018	0.044±0.010	-
Fish	Female	2	0.304±0.018	0.479±0.021	0.129±0.017	-
Control	Male	-	0.324±0.017	0.532±0.018	0.037±0.008	0.012±0.002
Fish	Male	-	0.289±0.018	0.508±0.025	0.108±0.015	0.004±0.001

Table 6.2: Results from LMMs and GLMMs for moving, immobility and hiding in control and fish treatment for paedomorphic newts. Statistically significant values are shown in bold.

Πίνακας 6.2: Αποτελέσματα των γραμμικών μοντέλων για την κινητικότητα, ακινησία και κρυπτική συμπεριφορά στα ενυδρεία ελέγχου και στα ενυδρεία παρουσία ψαριών για τους παιδομορφικούς τρίτωνες. Στατιστικά σημαντικές τιμές φαίνονται με έντονη γραφή.

Behavior	**Effects**	**F**	**NumDf**	**DenDf**	**P value**
Moving					
	Sex	5.714	1	10	**0.037**
	Treatment	7.533	1	10	**0.020**
	Sex*treatment	3.745	1	10	0.081
Immobility					
	Sex	4.332	1	10	0.062
	Treatment	0.885	1	10	0.368
	Sex*treatment	2.959	1	10	0.114
	Effects	**Chisq**	**Df**	**P value**	
Hiding					
	Sex	0.113	1	0.736	
	Treatment	6.634	1	**0.010**	
	Sex*treatment	0.039	1	0.843	

Treatment had a significant effect on hiding (GLMM: $\chi^2 = 6.634$, $p = 0.01$). In the fish treatment, females decreased movement and increased hiding and immobility, while males decreased movement and increased hiding (Figure 6.2).

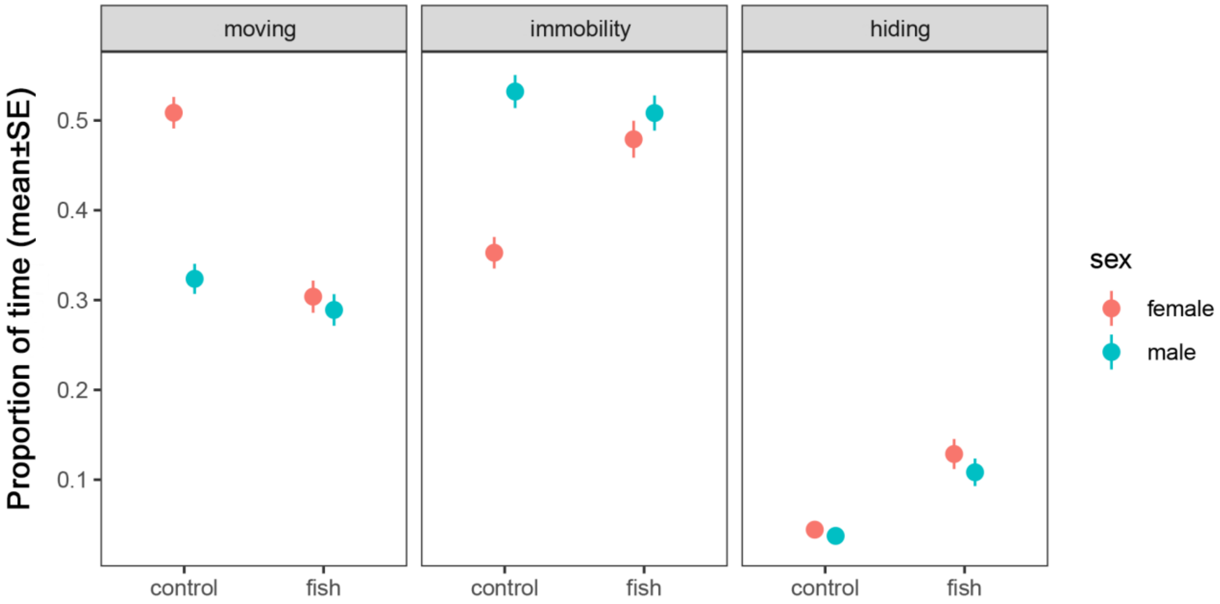

Figure 6.2: Proportion of time spent in moving, immobility and hiding in the two experimental treatments (control vs. mosquitofish) for female and male paedomorphic *Lissotriton graecus*.

Εικόνα 6.2: Αναλογία του χρόνου που αφιερώθηκε στην κινητικότητα, ακινησία και κρυπτική συμπεριφορά στα ενυδρεία ελέγχου και ενυδρεία παρουσία ψαριών για τους θηλυκούς και αρσενικούς παιδομορφικούς τρίτωνες *Lissotriton graecus*.

The presence of fish had a significant impact on the oviposition behavior of female newts (z = - 3.606, $p<0.001$), which laid considerably fewer eggs than in the absence of fish (Table 6.2).

Mosquitofish attacked frequently paedomorphic newts. I recorded a total of 72 attacks towards females and 40 attacks towards males over the whole observation period. A female paedomorphic newt, previously bitten by the mosquitofish, died after 3 days.

I found a significant main effect of treatment (GLMM: χ^2 = 7.753, p = 0.005), sex (GLMM: χ^2 = 4.473, p = 0.034) and their interaction (GLMM: χ^2 = 3.877, p = 0.048) on the feeding activity of newts. Pairwise comparisons of the significant interaction are summarized in Table 6.3.

Table 6.3: Pairwise comparisons obtained by "lsmeans package" (Tukey method) of feeding activity for paedomorphic newts in control and treatment with fish. Statistically significant values are shown in bold.

Πίνακας 6.3: Αποτελέσματα πολλαπλών συγκρίσεων, σύμφωνα με τη μέθοδο Tukey, της τροφοληψίας των παιδομορφικών τριτώνων στα ενυδρεία ελέγχου και στα ενυδρεία παρουσία ψαριών. Οι στατιστικά σημαντικές τιμές δίνονται με έντονη γραφή.

Pairwise comparisons		**Estimate**	**SE**	**d.f.**	**T ratio**	**P value**
Control Female	Fish Female	3.248	0.979	NA	3.316	**0.005**
Control Female	Control Male	2.715	0.942	NA	2.881	**0.020**
Control Female	Fish Male	3.522	1.028	NA	3.424	**0.003**
Fish Female	Control Male	-0.533	0.793	NA	-0.672	0.907
Fish Female	Fish Male	0.273	0.841	NA	0.325	0.988
Control Male	Fish Male	0.806	0.819	NA	0.985	0.758

Treatment and sex had a significant main effect in minimum foraging latency, χ^2 = 18.1, p<0.001 and χ^2 = 9.2, p = 0.002, respectively. The interactive effect of treatment and sex was statistically significant, χ^2 = 50.2, p<0.001. The minimum foraging latency differed significantly between the sexes in control and was affected by fish presence for females (Table 6.4). Females reduced their overall foraging behavior by minimizing their engagement in a feeding act and increasing their foraging latency (Figure 6.3). Male newts foraged less than females during the experiment (Figure 6.3) and their minimum foraging latency did not differ significantly between the treatments (Table 6.4).

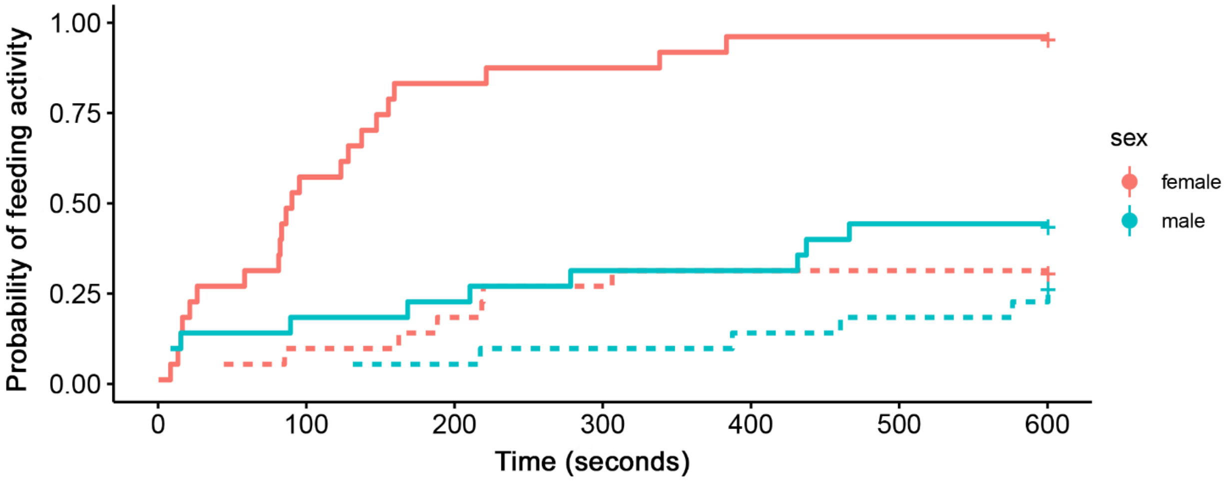

Figure 6.3: Probability of feeding activity in female and male paedomorphic newts, for control (solid line) and treatment with mosquitofish (dashed line) over the 10-min observation period.

Εικόνα 6.3: Πιθανότητα τροφοληψίας στους θηλυκούς και αρσενικούς παιδομορφικούς τρίτωνες, στα ενυδρεία ελέγχου (ενιαία γραμμή) και στα ενυδρεία με παρουσία ψαριών (διακεκομμένη γραμμή) κατά τη διάρκεια της 10’ παρατήρησης.

Table 6.4: Pairwise comparisons of the survival curves for feeding activity between sexes and treatments. Significantly values are indicated in bold.

Πίνακας 6.4: Αποτελέσματα πολλαπλών συγκρίσεων των καμπυλών επιβίωσης για την τροφοληψία μεταξύ των φύλων και των ενυδρείων. Οι στατιστικά σημαντικές τιμές δίνονται με έντονη γραφή.

Pairwise comparisons	**Log rank test**	
Sex*Treatment	χ^2	P
Female Control-Female Treatment	23.3	**<0.001**
Male Control-Male Treatment	1.9	0.165
Female Control-Male Control	16.4	**<0.001**
Female Treatment-Male Treatment	0.2	0.626
Female Control-Male Treatment	31.5	**<0.01**
Male Control-Female Treatment	0.7	0.395

Regarding metamorphosis, at the end of the 4-week experimental period, 75% of females and 100% of males metamorphosed in the presence of fish, whereas 0% of females and 60% of males metamorphosed in the control treatment. Sex and treatment had a significant main effect in time of metamorphosis, $\chi^2 = 5.5$, $p = 0.01$ and $\chi^2 = 6$, $p = 0.01$, respectively. The interactive effect of sex and treatment on timing of metamorphosis was statistically significant ($\chi^2 = 12.9$, $p = 0.004$). The timing of metamorphosis differed significantly between the sexes in both treatments and was affected by fish presence for females (Table 6.5). However, for male newts the timing of metamorphosis did not differ significantly between the treatments (Table 6.5), and they metamorphosed earlier than female newts both in the presence and absence of fish (Figure 6.4).

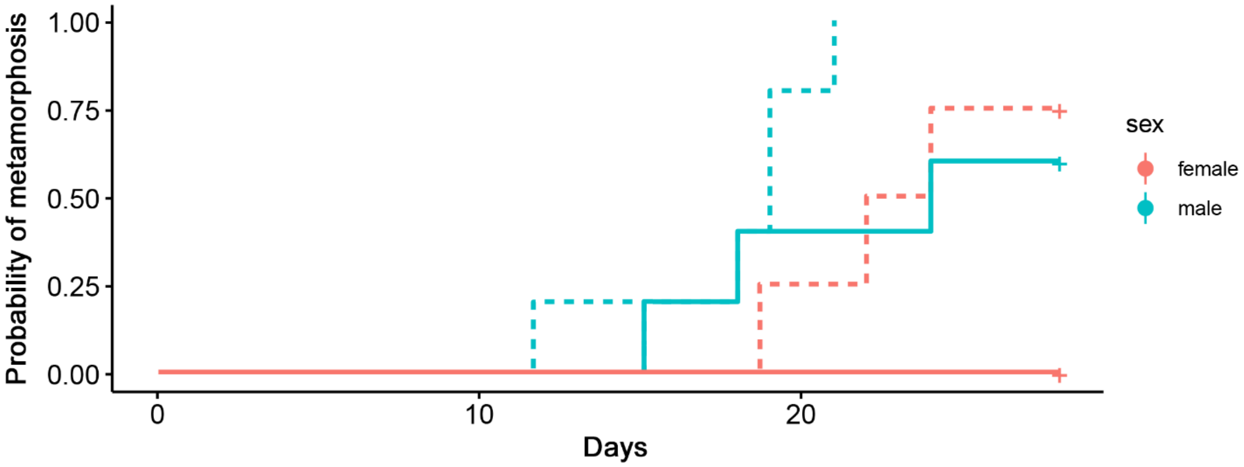

Figure 6.4: Probability of metamorphosis for female and male paedomorphic newts for control (solid line) and treatment with mosquitofish (dashed line) over the 4-week experimental period.

Εικόνα 6.4: Πιθανότητα μεταμόρφωσης στους θηλυκούς και αρσενικούς παιδομορφικούς τρίτωνες, στα ενυδρεία ελέγχου (ενιαία γραμμή) και στα ενυδρεία παρουσία ψαριών (διακεκομμένη γραμμή) κατά τη διάρκεια των 4 εβδομάδων του πειράματος.

Table 6.5: Pairwise comparisons of the survival curves between sexes and treatments on timing of metamorphosis. Significant values are indicated in bold.

Πίνακας 6.5: Αποτελέσματα πολλαπλών συγκρίσεων των καμπυλών επιβίωσης για το χρόνο μεταμόρφωσης μεταξύ των φύλων και των ενυδρείων. Οι στατιστικά σημαντικές τιμές δίνονται με έντονη γραφή.

Pairwise comparisons	**Log rank test**	
Sex*Treatment	χ^2	P
Female Control-Female Treatment	5.2	**0.02**
Male Control-Male Treatment	2.2	0.14
Female Control-Male Control	3.9	**0.04**
Female Treatment-Male Treatment	4.9	**0.02**
Female Control-Male Treatment	9.5	**0.002**
Male Control-Female Treatment	0.0	0.8

6.4 Discussion

In this study, I assessed the detrimental effects of an invasive fish on a native amphibian species from Greece. I found that mosquitofish presence had sublethal effects on paedomorphic newts, with consequences regarding their fitness. Newts exposed to the presence of mosquitofish reduced their mobility and their foraging behavior, as well as their oviposition rates. Most notably, the presence of fish induced an increase in the frequency of metamorphosis.

Animals spend time in crucial activities for survival, exposing themselves to visibility and potential risks. Several animal taxa adopt inactive behaviors by ‘‘freezing’’ in order to lower the possibility of an encounter with a threat (Skelly & Werner 1990, Chelini et al. 2009, Liden et al. 2010). This freezing response is a common anti-predator response in many amphibians (Skelly & Werner 1990, Gall & Mathis 2010).

In my study, both female and male paedomorphic newts decreased their mobility and overall activity, trying to become less detectable from the fish predator since they were prone to fish attacks. They considerably increased hiding, rather than adopting a freezing behavior, reducing that way the probability of detection and consequently predation (Stauffer & Semlitsch 1993). Such anti-predatory response can be advantageous in decreasing the risks of predation and injury. However, this response may have many effects on other essential activities such as reproduction and feeding (Bridges 2002, Stynoski & Noble 2012).

During reproduction, newts secrete pheromones and exhibit courtship displays (Malacarne & Giacoma 1986, Halliday 1990), processes that may attract potential predators. Minimizing sexual activity could imply a trade-off between predation risk and reproduction but costing several breeding opportunities. Recent studies show a similar reduction of sexual activity in amphibian species in the presence of potential fish predators (Cabrera-Guzmán et al. 2018, Winandy & Denoël 2019b). My study shows that mosquitofish affect oviposition in paedomorphic smooth newts causing a very high reduction in oviposition rates, since females stopped investing on producing eggs. The question remains open regarding courtship, as male newts had a low sexual display rate both in control and fish treatment during the experiment, suggesting the need of further research in order to disentangle any effects of mosquitofish in the sexual behavior of males. I did not test for fish consumption on eggs or aquatic larvae after hatching, which could be an additional detrimental factor. For instance, Vannini et al. (2018) showed increased mortality rates of newt larvae due to the presence of mosquitofish. Such effects could have long-term detrimental consequences on reproductive success and also contribute to the decline of local populations.

Throughout the experiment, paedomorphs reduced their overall foraging behavior in the presence of fish. This agrees with previous results where foraging activity of newts was also reduced in the presence of goldfish (Winandy & Denoël 2015, 2019a). On the long-term, this reduction of feeding activity could have harmful effects on growth rates, resulting in decreased fitness and survival rates.

Facultative paedomorphosis is an environmentally induced polymorphism (Whiteman 1994), which gives an advantage to pond-breeding salamanders and newts to exploit different ecological niches (Lejeune et al. 2018), and in some cases, an opportunity to escape potentially hostile habitats (Semlitsch 1987, Mathiron et al. 2017). Previous work on larvae of species with complex life cycles suggest an earlier transition to the terrestrial habitat, where more larvae metamorphose in smaller sizes to minimize the time spent in a poor aquatic habitat (Skelly & Werner 1990, Vonesh & Warkentin 2006, Cabrera-Guzmán et al. 2017, Vannini et al. 2018). Mosquitofish presence in the present study, led to a hostile environment for paedomorphic newts, since they reduced their activity, reproduction and feeding behavior, leading to an “escape”, facilitated by phenotypic transition. Thus, providing an experimental support to the several reports of disappearance of local paedomorphic populations after fish introduction (Denoël et al. 2005). An interesting pattern is that the behavioral responses of the paedomorphic newts are partially sex-specific. Sex-biased metamorphosis could reflect the sex-biased ratios in many local populations of newts (Sotiropoulos et al. 2017) and strengthen the importance of sex in the preservation of alternative evolutionary trajectories (Mathiron et al. 2017, Denoël et al. 2019c).

Notably, although microcosm experiments as the present one can be a powerful tool to disentangle complex behaviors, there are some limitations due to their simplicity and the manipulation of environmental parameters (Fraser & Keddy 1997). In this case, paedomorphs might show varying levels of intensity in their behavioral patterns or less prompt responses in the field, where there is a strong environmental complexity. Paedomorphic newt populations lost over 50% of their aquatic area of occupancy over a 10-year period due to fish introductions in Montenegro (Denoël et al. 2019a). Further field studies in natural aquatic ecosystems are necessary to investigate the extend of ecological phenomena such as predator–prey dynamics.

Despite that, my results offer some insight regarding newt conservation and highlight the detrimental effects of the increased fish introductions. Future conservation strategies should focus in preserving local amphibian populations and simultaneously regulating the mosquitofish populations and avoid overall fish introductions in freshwater communities.

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Chapter 7

Conclusions

Κεφάλαιο 7

Συμπεράσματα

The expression of facultative paedomorphosis in natural populations is regulated by many factors and complex underlying mechanisms, while the persistence of the polyphenis affected by environmental conditions. Here, the aim of the thesis was to investigate the evolutionary processes involved in a wild population of the Greek smooth newt where both paedomorphic and metamorphic individuals coexist and study their interaction with possible environmental stressors.

The main conclusions can be summarized:

- Facultative paedomorphosis seems to be maintained by fitness differences regarding the female newts, since females benefit from longer residence in the aquatic habitat by avoiding the costs of metamorphosis.
- Females with higher body size and better body condition, laid higher number of eggs and had more larvae that reached metamorphosis.
- There are significant sex-specific differences, since males do not show differences in body condition like female newts. Sexual selection plays a significant role in the maintenance of the polyphenism in newt populations.
- The genetic data reveal high gene flow and sexual compatibility between the alternative morphs. This strengthens the assumption that facultative paedomorphosis remains as a polyphenism and is maintained by fitness differences regarding each morphotype.
- Specific genes were found to be associated with each morphotype, contributing to the genetic basis of the polygenic trait of paedomorphosis in the Greek smooth newt.
- The genes found in the present study are involved in metabolic processes and stress response, highlighting the importance of energy and stress in phenotypic shifts.
- Body size is an important fitness trait in newts, and it is correlated with fecundity. This quantitative polygenic trait is associated with genes involved in cell proliferation.
- Genotype differences in the trait-associated loci were found between the alternative morphotypes, revealing the importance of specific polymorphisms in the genetic basis of fitness-related traits.
- Environmental stressors influence the persistence of the paedomorphic phenotype, which is negatively associated with disturbances in the aquatic habitat, such as fish introductions and pond desiccation.

- Invasive fish species have sublethal effects on paedomorphic newts, with consequences regarding their fitness. Newts reduce their mobility and their foraging behavior, as well as their oviposition rates in the presence of fish.
- Higher rates of metamorphosis are observed under environmental stressors with severe consequences in the persistence of the alternative morphotype.
- Sex-biased responses are observed in metamorphosis rates and behavioral changes, confirming the "male escape hypothesis" and emphasize the importance of sex in the expression of polyphenisms.

Phenotypic changes in respect to environmental cues or signals, increase fitness advantage of organisms, leading to adaptation and facilitating evolution of complex phenotypes. Facultative paedomorphosis is a major evolutionary process highly connected to the environment and is maintained due to fitness acting differently in each sex regarding the prevailing environmental conditions. The above results and conclusions contribute significantly to the existing knowledge of the proximate mechanisms of the expression of facultative paedomorphosis in newts and the genetic basis of complex phenotypes. Future research should focus in measuring the fitness consequences of each phenotype in each sex during their lifetime and in several developmental stages. Further genetic examination of the identified candidate loci on other newt population and species exhibiting facultative paedomorphosis, can unravel the genetic basis of the polygenic trait and possible underlying mechanisms acting on functional genes that mediate adaptation and plasticity in newts. The thesis also offers insight regarding newt conservation, hence contributing on possible management actions for the preservation of paedomorphosis, an important alternative ontogenetic trajectory.

Extended summary

Many salamandrid species exhibit paedomorphosis, a polyphenism in which sexually mature individuals retain larval external morphology, such as gill slits and external gills. Facultative paedomorphosis is an environmentally induced polyphenism and its manifestation can be predicted by fitness differences, where one morphotype might have greater fitness advantage over the other in the prevailing environmental conditions.

The aim of this dissertation is to explore the underlying mechanisms on the expression and persistence of facultative paedomorphosis in newts. The study focuses on a natural population of the Greek smooth newt (*Lissotriton graecus*) exhibiting facultative paedomorphosis, and implements behavioral and ecological experiments, along with genetic tools (**Chapters 2, 3 & 4**). In addition, aim of this dissertation is to provide an estimate of the viability of the studied population on the long-term, along with the possible effects of different environmental stressors in the persistence of facultative paedomorphosis in newts (**Chapters 5 & 6**).

Chapter 2 explores the proximate mechanisms of facultative paedomorphosis, by using body condition and reproductive components as fitness proxies. Fitness is a key concept in evolutionary biology where each given genotype or phenotype contributes differently to the gene pool of the next generation. More specifically, courtship behaviors and mating preferences were observed, along with egg production, larval development, and offspring viability of female newts, to explore potential fitness differences between the two phenotypes. The experiments showed that body size has a significant positive effect in fecundity. At the same time, paedomorphic female newts exhibited higher body condition than metamorphic females and produced more surviving larvae and had a better outcome in reproduction. Male newts did not show differences in body condition but showed preferences in courtship behavior towards paedomorphic female newts probable due to differences in fitness and higher abundances in the studied population.

Despite the significance of phenotypic plasticity in the evolution and ecology of natural populations and the ongoing development of new genomic tools, the underlying genetic basis remains unresolved. **Chapter 3** explores levels of genetic divergence between the two morphotypes by obtaining thousands of genetic markers through dd-RAD sequencing and provides insights into the underlying genetic mechanisms of facultative paedomorphosis. Based on the obtained SNP data, low genetic divergence between the two alternative

morphotypes and similar levels of gene diversity on neutral markers were observed. Despite the observed high gene flow, an *Fst* approach for outliers detected candidate loci putatively associated with the alternative morphs that mapped to four genes. These identified genes have functional roles in metabolic processes, energy production and stress response.

Utilizing the results of **Chapter 2**, where body size was found to be positively correlated with fecundity and the thousands of marker loci obtained through dd-RAD sequencing in **Chapter 3**, the aim of **Chapter 4** was to explore the genetic basis of the quantitative phenotypic trait of body size and detect any heterozygosity-fitness correlations (HFCs) that may arise in the population. The results of the genome-wide association study identified loci highly associated with body size and revealed candidate genes putatively associated with epithelial cell proliferation. Cell proliferation, the increase in the number of cells, is an important factor from embryogenesis to tissue repair and growth and is regulated by complex mechanisms. Regarding levels of heterozygosity, I did not observe any correlation of fitness-related traits with genome-wide heterozygosity, however genotypic differences between the two morphotypes were observed in the identified genes that are correlated with body size.

Paedomorphic population depend on both aquatic and terrestrial habitat and are prone to alterations of the environmental conditions. **Chapter 5** aims to predict the extinction risk of the studied paedomorphic population, and to investigate the sensitivity of the paedomorphic phenotype to pond desiccation and fish introductions, that are considered as the most environmental stressful factors for newt populations. According to the population viability analysis, both these environmental factors lead to the extinction of the paedomorphic phenotype within the next 25 years, while sex-specific differences in the persistence of the paedomorphic morphotype were evident, with male newts metamorphosing earlier than females in stressful environmental conditions, probable due to differences in life history traits.

In Epirus prefecture, NW Greece, several cases of facultative paedomorphosis occurrence have been recorded, while several lakes and ponds in the region have been stocked with fish for recreational fishing, rising concerns for the negative impacts to the paedomorphic populations. To this end, **Chapter 6** aims to investigate and quantify the impact of the most invasive fish species, the eastern mosquitofish (*Gambusia holbrooki*), on paedomorphic newts as a result of direct physical disturbance and/or indirect modification of their behavior, and the effects on metamorphosis rate as well. Mosquitofish had a negative impact on paedomorphic newts by inducing both behavioral and phenotypic changes. Paedomorphic newts exhibited avoidance behavior and higher metamorphosis rates in the presence of fish. Both female and male newts responded by decreasing mobility and foraging activity, and females stopped investing in egg-laying. Such effects could have long-term detrimental consequences on reproductive success and contribute to the decline of focal populations.

The combined results of the present study conform that facultative paedomorphosis remains as a polymorphism in the studied population and converge to the "paedomorphosis advantage hypothesis", since paedomorphic female newts exhibit higher body condition and fecundity. Male newts are becoming choosier, and according to the courtship behavior experiment, it seems they choose female paedomorphs rather than metamorphs probably due to the fitness advantages and higher abundance in the population. Although, the lack of genetic differentiation conforms sexual compatibility between the alternative morphotypes, it strengthens the hypothesis that facultative paedomorphosis remains as a polyphenism to reduce intra-population competition.

Additionally, the study offers valuable insights into the genetic basis of paedomorphosis, since the identified genes putatively associated with each morphotype have a significant role in energy production and stress response, important metabolic processes in phenotypic changes. At the same time, body size is an important fitness proxy and substantial differences were found between the two morphotypes. The identified genes and genetic variants associated with the quantitative trait of body size offer the possibility for further examination of genetic polymorphisms at the specific loci and their association with the different sizes observed in the Greek smooth newt.

Lastly, the persistence of facultative paedomorphosis is prone to environmental changes, while introduction of alien fish species, specifically, has detrimental effects on paedomorphic newt populations leading to the extirpation of the alternative phenotype. Especially, in the region of Epirus, where several cases of paedomorphosis have been reported and is an important area for the conservation of alternative ontogenetic trajectories like paedomorphosis, it is necessary to plan effective management actions for the protection and the persistence of paedomorphic newt populations and their habitats.

Εκτεταμένη Περίληψη

Πολλά είδη ουρόδηλων αμφιβίων εμφανίζουν παιδομόρφωση. Η παιδομόρφωση αποτελεί χαρακτηριστικό παράδειγμα φαινοτυπικής πλαστικότητας όπου τα αναπαραγωγικά ώριμα άτομα διατηρούν χαρακτήρες των προνυμφικών σταδίων, ενώ αποτελεί ένα σημαντικό εξελικτικό μονοπάτι για τη μείωση του ενδο-ειδικού ανταγωνισμού. Η παραγωγή αυτών των εναλλακτικών φαινοτύπων είναι αποτέλεσμα της συνδυαστικής δράσης περιβαλλοντικών και γενετικών παραγόντων, και σχετίζεται με το κόστος και τα οφέλη της επιβίωσης του ατόμου στο εκάστοτε περιβάλλον.

Στόχος της παρούσας διατριβής είναι η διερεύνηση των μηχανισμών που εμπλέκονται στην εμφάνιση της περιστασιακής παιδομόρφωσης στους τρίτωνες. Η διατριβή εστιάζει στη μελέτη της αρμοστικότητας των εναλλακτικών φαινοτύπων σε έναν άγριο πληθυσμό του ελληνικού κοινού τρίτωνα (*Lissotriton graecus*) ο οποίος εμφανίζει περιστασιακή παιδομόρφωση, μέσω μιας σειράς πειραμάτων συμπεριφοράς και οικολογίας, καθώς και με τη χρήση γενετικών εργαλείων (**Κεφάλαια 2, 3 & 4**). Παράλληλα, καθώς οι παιδομορφικοί πληθυσμοί είναι ιδιαίτερα ευάλωτοι στις περιβαλλοντικές αλλαγές, στόχος τη διατριβής είναι η μελέτη των επιπτώσεων συγκεκριμένων περιβαλλοντικών πιέσεων στην εκδήλωση και διατήρηση της παιδομόρφωσης (**Κεφάλαια 5 & 6**).

Το **Κεφάλαιο 2** διερευνά τους εγγύς μηχανισμούς της περιστασιακής παιδομόρφωσης, χρησιμοποιώντας τον σωματικό δείκτη και την αναπαραγωγή ως συνιστώσες της αρμοστικότητας. Η μέτρηση των διαφορών της ατομικής αρμοστικότητας είναι ουσιώδης για την κατανόηση της εξέλιξης και της διατήρησης εναλλακτικών οντογενετικών μονοπατιών. Συγκεκριμένα μελετήθηκαν οι ερωτοτροπικές συμπεριφορές, καθώς και ο αριθμός των αυγών που εναπόθεσαν τα θηλυκά άτομα και ο αριθμός των προνυμφών που εκκολάφθηκαν επιτυχώς, προκειμένου να διερευνηθούν πιθανές διαφορές μεταξύ των δύο φαινοτύπων. Σύμφωνα με τα αποτελέσματα, το μέγεθος του σώματος βρέθηκε να έχει σημαντική θετική συσχέτιση με την αναπαραγωγή, και ταυτόχρονα, τα παιδομορφικά θηλυκά φαίνεται να παρουσιάζουν καλύτερη φυσική κατάσταση σε σχέση με τα μεταμορφωμένα. Η κατάσταση του σώματος συσχετίζεται με την αποθήκευση λιπιδίων και ενέργειας, τα οποία είναι ζωτικής σημασίας για την γονιμότητα και την αναπαραγωγή. Τα παιδομορφικά θηλυκά γέννησαν προνύμφες με υψηλότερη βιωσιμότητα, συμβάλλοντας στην πρώτη γενιά. Όσον αφορά στα αρσενικά, δεν παρατηρήθηκαν διαφορές στη σωματική κατάσταση μεταξύ των εναλλακτικών μορφών, όμως οι ερωτοτροπικές συμπεριφορές τους φανερώνουν επιλογή της παιδομορφικής μορφής των θηλυκών ατόμων, πιθανώς λόγω της αφθονίας τους στον πληθυσμό και της αυξημένης αναπαραγωγικής αρμοστικότητας.

Η παιδομόρφωση αποτελεί ένα παράδειγμα φαινοτυπικού πολυμορφισμού, και παρά τη σημασία της φαινοτυπικής πλαστικότητας στην εξέλιξη και την οικολογία των φυσικών πληθυσμών και τη συνεχιζόμενη ανάπτυξη νέων γονιδιωματικών εργαλείων, η υποκείμενη γενετική βάση της παραμένει ακόμα άγνωστη. Στο **Κεφάλαιο 3**, εξετάστηκαν οι υποκείμενοι μηχανισμοί γενετικής και φαινοτυπικής διαφοροποίησης μεταξύ των εναλλακτικών μορφών στον πληθυσμό μελέτης, χρησιμοποιώντας τη μέθοδο dd-RAD, με στόχο την ανίχνευση πιθανής υποκείμενης γενετικής δομής στον πληθυσμό τριτώνων όπου συνυπάρχουν και οι δύο μορφότυποι, τη διερεύνηση της γενετικής ποικιλότητας του πληθυσμού στο σύνολό του και σε σχέση με κάθε μορφότυπο, και την ανίχνευση υποψήφιων γενετικών τόπων που σχετίζονται με την κάθε μορφή. Με βάση τα ληφθέντα δεδομένα SNP, παρατηρήθηκε χαμηλή γενετική διαφοροποίηση μεταξύ των δύο εναλλακτικών μορφών και παρόμοια επίπεδα γονιδιακής ποικιλότητας σε ουδέτερους δείκτες. Παράλληλα, χρησιμοποιώντας μια προσέγγιση *Fst* για ακραίες τιμές SNP, εντοπίστηκαν 35 γενετικοί τόποι που πιθανώς συσχετίζονται με τις εναλλακτικές μορφές και χαρτογραφήθηκαν σε συνολικά τέσσερα γονίδια. Τα αναγνωρισμένα αυτά γονίδια φαίνεται να έχουν λειτουργικούς ρόλους σε σημαντικές μεταβολικές διεργασίες, όπως η παραγωγή ενέργειας και πρωτεϊνών, και η ανταπόκριση σε στρεσσογόνους παράγοντες.

Αξιοποιώντας τα αποτελέσματα του **Κεφαλαίου 2**, όπου βρέθηκε να υπάρχει θετική συσχέτιση του σωματικού μεγέθους των θηλυκών τριτώνων με την αναπαραγωγική επιτυχία, και του **Κεφαλαίου 3** όπου αποκτήθηκαν χιλιάδες μοναδιαίες θέσεις πολυμορφισμού (SNPs), απώτερος στόχος του **Κεφαλαίου 4** είναι η διερεύνηση του γενετικού υπόβαθρου ενός πολυγονιδιακού φαινότυπου και η πιθανή ύπαρξη συσχετίσεων της γενετικής ποικιλότητας με την αρμοστικότητα. Το μέγεθος αποτελεί έναν σημαντικό φαινοτυπικό χαρακτήρα στα αμφίβια που επηρεάζεται από τη συνδυασμένη έκφραση πολλών γονιδίων. Μέσω της μελέτης συσχέτισης γονιδιώματος με το σωματικό μέγεθος, εντοπίστηκαν πολυμορφισμοί που συσχετίζονται με το σωματικό μέγεθος, και χαρτογραφήθηκαν συνολικά τέσσερα γονίδια που συμμετέχουν στον πολλαπλασιασμό κυττάρων, σημαντική λειτουργία του οργανισμού για την δημιουργία νέων κυττάρων και οργάνων, και την ανάπτυξη του σώματος. Παράλληλα, βρέθηκαν γονοτυπικές διαφορές μεταξύ των εναλλακτικών μορφών που συνδέονται με το μέγεθος. Η γενετική ποικιλότητα όπως εκφράζεται από την παρατηρούμενη ετεροζυγωτία δεν βρέθηκε να συσχετίζεται με την αρμοστικότητα όπως εκφράζεται από το σωματικό μέγεθος και την αναπαραγωγική ικανότητα.

Οι παιδομορφικοί πληθυσμοί, λόγω της στενής εξάρτησής τους από το υδάτινο μέσο, καλούνται να αντιμετωπίσουν σοβαρές περιβαλλοντικές απειλές. Στο **Κεφάλαιο 5**, γίνεται εκτίμηση της πιθανότητας εξαφάνισης του τοπικού πληθυσμού, καθώς και διερεύνηση της πιθανότητας εξαφάνισης του παιδομορφικού μορφότυπου, αναλύοντας δημογραφικά δεδομένα, ποσοστά βιωσιμότητας και γονιμότητας, γενετικά δεδομένα, καθώς και γνωστούς ρυθμούς μεταμόρφωσης. Μελετήθηκαν σενάρια που αποτελούν τις βασικότερες περιβαλλοντικές απειλές για πληθυσμούς παιδομορφικών τριτώνων, όπως είναι η εισαγωγή ψαριών και η ξήρανση του υδάτινου ενδιαιτήματος. Σύμφωνα με την ανάλυση βιωσιμότητας, και οι δύο απειλές θα οδηγήσουν στην εξαφάνιση του πληθυσμού και της παιδομορφικής μορφής μέσα στα επόμενα 25 χρόνια. Η εισαγωγή ψαριών φαίνεται πως αυξάνει πολύ την πιθανότητα εξαφάνισης, ενώ παρατηρούνται και διαφορές μεταξύ των δύο φύλων, με τα αρσενικά να δέχονται τη μεγαλύτερη πίεση.

Στην ευρύτερη περιοχή του νομού Ιωαννίνων έχουν καταγραφεί πέντε πληθυσμοί τριτώνων που εμφανίζουν περιστασιακή παιδομόρφωση. Ωστόσο, στην περιοχή της λεκάνης των Ιωαννίνων, έχει διαπιστωθεί συστηματική εισαγωγή ψαριών από ιδιώτες σε μεγάλο αριθμό υδατοσυλλογών, με στόχο τη δημιουργία θέσεων για ερασιτεχνικό ψάρεμα. Στόχος του **Κεφαλαίου 6** είναι η μελέτη των πιθανών διαταραχών στη συμπεριφορά παιδομορφικών τριτώνων παρουσία κουνουπόψαρων (*Gambusia holbrooki*), ενός ιδιαίτερα εισβλητικού είδους στην περιοχή, καθώς και κατά πόσον η παρουσία τους προάγει ή επιταχύνει το ρυθμό μεταμόρφωσης των παιδομορφικών ατόμων. Σύμφωνα με τα αποτελέσματα της μελέτης, διαπιστώθηκε πως τα παιδομορφικά άτομα μειώνουν την κινητικότητά τους και αυξάνουν την κρυπτική τους συμπεριφορά. Οι θηλυκοί τρίτωνες μείωσαν την ωοαπόθεση αυγών, και παράλληλα καταγράφηκαν πολλές επιθέσεις των κουνουπόψαρων προς τους τρίτωνες. Η τροφοληψία των παιδομορφικών τριτώνων μειώθηκε με την παρουσία των ψαριών, και παρατηρήθηκε αυξημένος ρυθμός μεταμόρφωσης. Η αποφυγή σημαντικών συμπεριφορών (αναπαραγωγή, τροφοληψία, ερωτοτροπία), μπορεί να οδηγήσει σε μειωμένους ρυθμούς ανάπτυξης, αναπαραγωγικής επιτυχίας και αρμοστικότητας, με επακόλουθο αποτέλεσμα τη μείωση των τοπικών πληθυσμών και την εμφάνιση κατακερματισμένων κατανομών.

Τα αποτελέσματα της παρούσας έρευνας υποδεικνύουν πως η περιστασιακή παιδομόρφωση παραμένει ως φαινοτυπικός πολυμορφισμός στον υπό μελέτη πληθυσμό, και προκρίνουν την «Υπόθεση του Πλεονεκτήματος της Παιδομόρφωσης» (“paedomorphosis advantage hypothesis”), καθώς τα παιδομορφικά θηλυκά άτομα εμφανίζουν μεγαλύτερη αρμοστικότητα, όπως εκτιμήθηκε από το σωματικό μέγεθος και την αναπαραγωγική επιτυχία. Η αυξημένη αναπαραγωγική επιτυχία και αφθονία των παιδομορφικών θηλυκών οδηγεί στην επιλογή τους από τα αρσενικά άτομα. Παρ ’όλα αυτά, η έλλειψη γενετικής διαφοροποίησης μεταξύ των εναλλακτικών φαινοτύπων φανερώνει την απρόσκοπτη γονιδιακή ροή, και επιβεβαιώνει πως η παιδομόρφωση παραμένει ως πολυφαινισμός και ως ένας τρόπος μείωσης του ενδο-ειδικού ανταγωνισμού στον πληθυσμό μελέτης.

Παράλληλα, η παρούσα μελέτη προσφέρει σημαντικές πληροφορίες για το γενετικό υπόβαθρο της παιδομόρφωσης, καθώς τα υποψήφια γονίδια που σχετίζονται με κάθε μορφή, συμμετέχουν στην παραγωγή ενέργειας και στην απόκριση στο στρες, σημαντικές μεταβολικές διεργασίες κατά τη διάρκεια φαινοτυπικών αλλαγών. Ταυτόχρονα, το σωματικό μέγεθος αποτελεί μία σημαντική συνιστώσα αρμοστικότητας, γεγονός που υποστηρίζεται από την θετική συσχέτιση με την αναπαραγωγική ικανότητα των θηλυκών. Τα αναγνωρισμένα γονίδια και οι γονοτυπικές διαφορές που βρέθηκαν στο φαινοτυπικό χαρακτήρα του σωματικού μεγέθους, φανερώνουν τη σημασία των γενετικών πολυμορφισμών, και η περαιτέρω μελέτη τους μπορεί να ρίξει φως στο διαφορετικό μέγεθος που εμφανίζουν οι δύο μορφές.

Τέλος, η εκδήλωση της παιδομόρφωσης είναι ιδιαίτερα ευαίσθητη στις περιβαλλοντικές αλλαγές, ενώ η εισαγωγή ξενικών ειδών ψαριών μπορεί να έχει ζημιογόνες επιπτώσεις στους πληθυσμούς τριτώνων και να οδηγήσει στην εξαφάνιση του εναλλακτικού φαινοτύπου. Ιδιαίτερα στην Ήπειρο, που αποτελεί ένα «θερμό σημείο» βιοποικιλότητας και σημαντική περιοχή για τη διατήρηση της προσαρμοστικής ενδο-ειδικής ποικιλότητας και των εναλλακτικών στρατηγικών διαβίωσης, κρίνεται απαραίτητος ο σχεδιασμός αποτελεσματικών διαχειριστικών δράσεων για την προστασία και διατήρηση των παιδομορφικών πληθυσμών και των οικοτόπων στους οποίους διαβιούν.

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Ευρετήριο Εικόνων

Ευρετήριο Πινάκων