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Assessment of the Endangered Species *Podarcis carbonelli* on a Microgeographic Scale: A Molecular, Morphological and Physiological Approach

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ASSESSMENT OF THE ENDANGERED SPECIES *PODARCIS CARBONELLI* ON A MICROGEOGRAPHIC SCALE: A MOLECULAR, MORPHOLOGICAL AND PHYSIOLOGICAL APPROACH

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ASSESSMENT OF THE ENDANGERED SPECIES *PODARCIS CARBONELLI* ON A MICROGEOGRAPHIC SCALE: A MOLECULAR, MORPHOLOGICAL AND PHYSIOLOGICAL APPROACH

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ASSESSMENT OF THE ENDANGERED SPECIES *PODARCIS CARBONELLI* ON A MICROGEOGRAPHIC SCALE: A MOLECULAR, MORPHOLOGICAL AND PHYSIOLOGICAL APPROACH

Name: Maria Clara Figueirinhas do Amaral Date: August 2009 Pages:88
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The lizard *Podarcis carbonelli* is an endangered species endemic to the Iberian Peninsula. One location where this species occurs is at the Berlengas Natural Preserve, an Atlantic archipelago off the coast of Portugal. These island populations are geographically separated from nearby mainland populations. The fundamental question is, are these insular individuals distinct from the mainland populations? Four localities were chose for comparison: two island populations and two nearby coastal populations. We assessed this question using three distinct approaches: molecular, morphological and physiological approach. We sequenced the 12S RNA, the mtDNA Control Region and the 7th intron of the β-fibrinogen gene and determined genetic diversity values as well as several parameters of population structure and differentiation. Individuals from these populations were also measured for several biometric characters and their blood lactate concentration was sampled.

There was no genetic variation in both the mtDNA regions analyzed. The nuclear intron revealed high levels of genetic variation, with islands having in general lower values than the mainland regions. The four populations sampled had low levels of divergence; the populations of Berlenga and Peniche were the most distinct and the populations of Farilhão and Baleal were the most similar from the four populations sampled.

Morphometric analyses revealed a different pattern of similarity among populations with the population of Farilhão being the only population statistically distinct from all other populations based on mass and SVL. Furthermore, island populations were in general more similar to each other than to mainland populations, with the exception of Berlenga males which in size are more similar to the Peniche males. The analysis of the blood lactate concentration revealed that the population of Peniche has significantly lower blood lactate levels than the populations of Farilhão and Berlenga.

The lack of genetic differentiation found in the populations under study is most likely due to the recent divergence of these populations. Furthermore, the genetically most different populations (Berlenga and Peniche) are not the most distinct in terms of morphology, particularly the males. This suggests that genetic drift, the most likely mechanism behind the genetic differentiation seen, is not responsible for the morphological differences observed. The morphological differences seen can be attributed to: a possible difference in age of the individuals in each population; mechanisms of natural selection that are favoring specific phenotypes in each of the populations, or phenotypic plasticity. The differences in blood lactate levels found between the population of Peniche and the island populations can be attributed to differences in predatory pressure or home range size.

It is suggested that the island populations are closely monitored due to their likely isolation, low mtDNA diversity and possible higher predatory pressure than initially predicted.

I. **Introduction**

A. *Podarcis carbonelli*

The wall lizard *Podarcis carbonelli* (Reptilia, Lacertidae), first described in 1981 by Pérez-Mellado, was formally described as a species by Paulo Sá-Sousa and D. James Harris (2002).

Podarcis carbonelli, endemic to the Iberian Peninsula, is a lizard with an average snout-vent length less than 63.5 mm (Figures 1 and 2). Carbonell's Wall lizard exhibits sexual dimorphism both in the dorsal coloration during the mating season and the size of the individuals. During the mating season, the dorsum of males is bright green with brown or black patterns forming longitudinal reticulate stripes along the body. Females do not possess this bright green color, and their dorsal coloration is brown with a reticulate pattern of dark brown or black (Sá-Sousa, 2004).

In Portugal, this species is typically located in areas with Atlantic climatic influences, *e.g.* along the coastal line of Portugal up to the Douro River. *Podaris carbonelli* is also found in the Berlengas archipelago along the Portuguese west coast (Figure 3) (Sá-Sousa and Harris, 2002; Sá-Sousa, 2004). In Spain, it is only found in two areas: the provinces of Salamanca and Cáceres and in an isolated location in Doñana.

Podarcis carbonelli is listed as Endangered (EN) on the International Union for Conservation of Nature 2007 Red List of threatened species and Vulnerable (VU) in the Portuguese Vertebrates Red Book. Its major threats are habitat degradation and loss due to fires, wood plantations, and tourism which have increased significantly in the areas where this species lives (IUCN, 2009; ICNB, 2006).

Figure 1. *Podarcis carbonelli* male from the population of Peniche with breeding season coloration.

Figure 2. *Podarcis carbonelli* female from the population of Peniche.

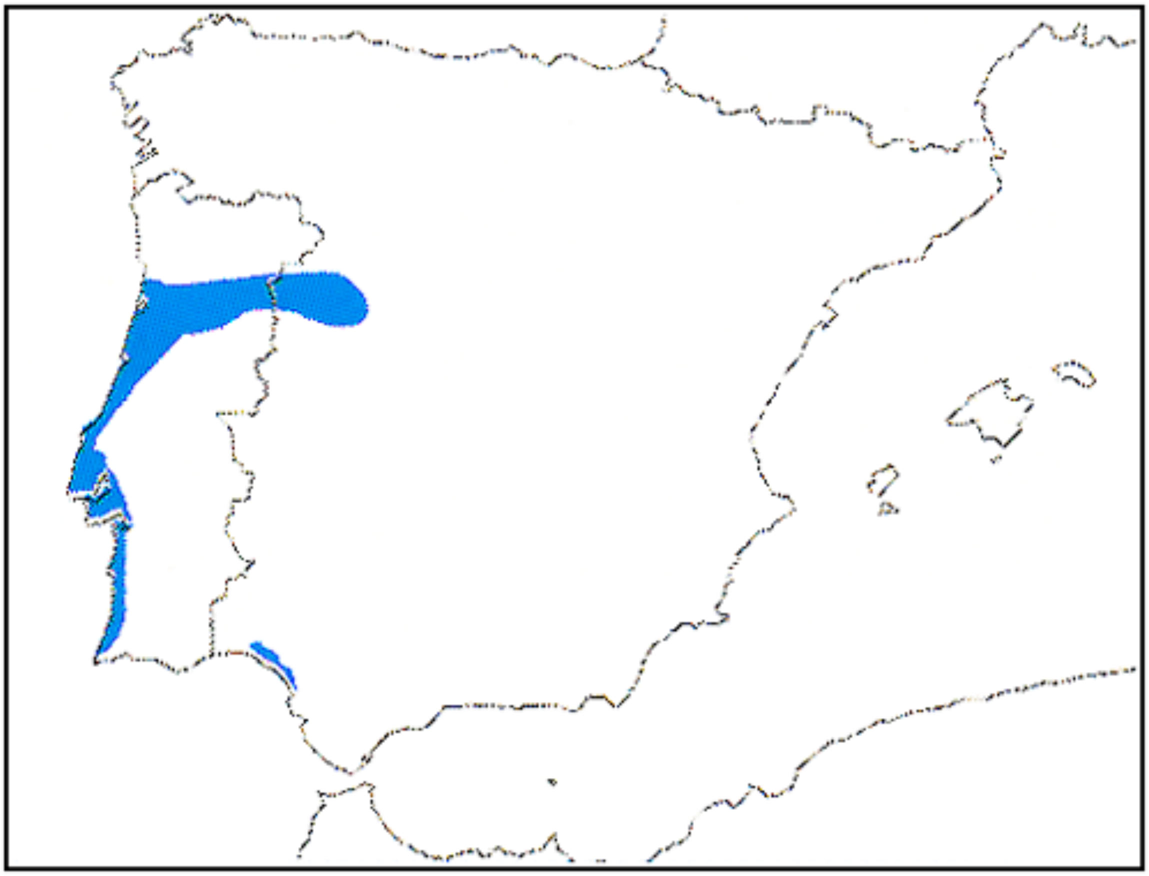

Figure 3. Current distribution of *P. carbonelli* in Portugal and Spain (modified from Pleguezuelos, 2004).

Another possible threat for this species is the documented occurrence of hybridization between *P. carbonelli* and another wall lizard, *Podarcis bocagei* on the northern limit of *P. carbonelli*'s distribution (Pinho et. al, in press).

The current distribution of *P. carbonelli* is highly patchy. Some authors attribute the current distribution to climatic events that took place during the Pleistocene and Holocene. One observation that corroborates this hypothesis is the fact that the southern populations are now more rare and locally distributed when compared to the northern populations, probably due to the fact that the southern environment is becoming progressively less suitable to the species (Sá-Sousa, 2004).

B. Evolutionary history of *Podarcis carbonelli*

Using mitochondrial DNA sequences, *P. carbonelli* is estimated to have been present in the Iberian Peninsula since around 500,000 to 313,000 years ago (Pinho et. al, 2007). A previously published study on the phylogeny of Iberian and North African wall lizards places *P. carbonelli* as a sister species of *P. hispanica*, type 2, of South Western Iberia (Pinho et. al, 2006). The information from mtDNA sequences reveals clear geographical structure across the distribution of *P. carbonelli* (Pinho et. al, 2007). However, another study based on intron sequencing has demonstrated that differentiation of Iberian species of *Podarcis* is low, although according to mtDNA it appears to be high. Furthermore, while mtDNA sequences portray the species as monophyletic, the nuclear sequences fail to represent the species as such (Pinho et. al, 2008).

C. Exercise metabolism in reptiles

Several studies on lizard metabolism have shown that anaerobic metabolism plays an important role in the physiology of this group (Bennett, 1973; Donovan and Gleeson, 2001). Most reptiles have the capacity for short, but very intense, bursts of activity that are fueled 80% to 90% by anaerobic metabolic pathways (Bennett and Licht, 1972; Bennett et. al, 1975). Research with reptiles has demonstrated that intense activity is associated with glycogenolysis and with an increase in blood lactic acid (Gleeson and Dalessio, 1989; Bennett, 1994). Furthermore, reptiles are known to survive under anoxic conditions by relying on anaerobic pathways (Bennett, 1978). The fact that plasma levels of lactate dehydrogenase are 10 to 20-fold greater in lizards vs. mammals, and the glycolytic enzymes such as phosphofructokinase presenting high levels in the muscle of iguanid lizards, suggests that reptiles are particularly capable of relying on anaerobiosis (Bennett, 1972; Bennett and Licht, 1972).

Anaerobic metabolism yields a small amount of energy when compared to the aerobic metabolism as the degradation of glycogen and glucose to lactic acid only yields 2 ATP molecules per glucose molecule. This process is not considered very efficient when compared to the approximate 36 ATP molecules obtained from the aerobic oxidation of glucose (Mader, 2003). Furthermore, anaerobic metabolism can have adverse effects on blood pH and blood oxygen carrying capacity due to the production of lactic acid. With that said, anaerobic metabolism is a quicker source of energy, with a shorter turn-around time as it depends only on molecules present in the muscle to produce energy (Bennett and Licht, 1972; Bennett, 1994).

When a reptile undergoes intense muscle activity, lactate is produced in the muscles as a result of anaerobic metabolism. Although muscle glycogen stores are replenished after intense exercise, the location where lactate is catabolized is still unknown. One hypothesis states that lactate is transported into the blood and is catabolized in the liver. Alternatively, it has also been demonstrated that lactate can undergo gluconeogenesis in the muscle, giving rise to glucose and glycogen, or be oxidized into CO_2 in the muscle, or both (Gleeson and Dalessio, 1989).

The possible pathways for lactate removal after intense exercise studied in reptiles have also been described in mammals. In mice that have been under intense muscle activity, 60 to 75% of the lactate produced is oxidized into CO_2 and only 11% is converted back to glucose and glycogen through the Cori cycle (Gleeson and Dalessio, 1989); Gleeson and DaLession (1989) have demonstrated with the use of isotopes in the desert iguana, that 50% of the lactate produced after intense activity is converted back to glucose and glycogen (conservative estimate) and only a small fraction of lactate is oxidized into CO_2 (Figure 4). In this work, the authors also found the role of hepatic gluconeogenesis to be minimal and the majority of lactate removal appeared to happen in the muscle.

The above mentioned studies have all been conducted in lizards from the family Iguanidae. Current information regarding lactate metabolism in lizards from the family Lacertidae is slight, but there are some behavioral data regarding activity of lacertids in their natural environment. Diaz and Cabezas-Diaz (2004) have studied behavioral mechanisms of the lacertid *Psammodromus algirus* and have described shuttling between different microhabitats as an important component of this organism's thermoregulation. Furthermore, lizards of the species *P. carbonelli* also resort to bursts of speed when necessary to escape from predators.

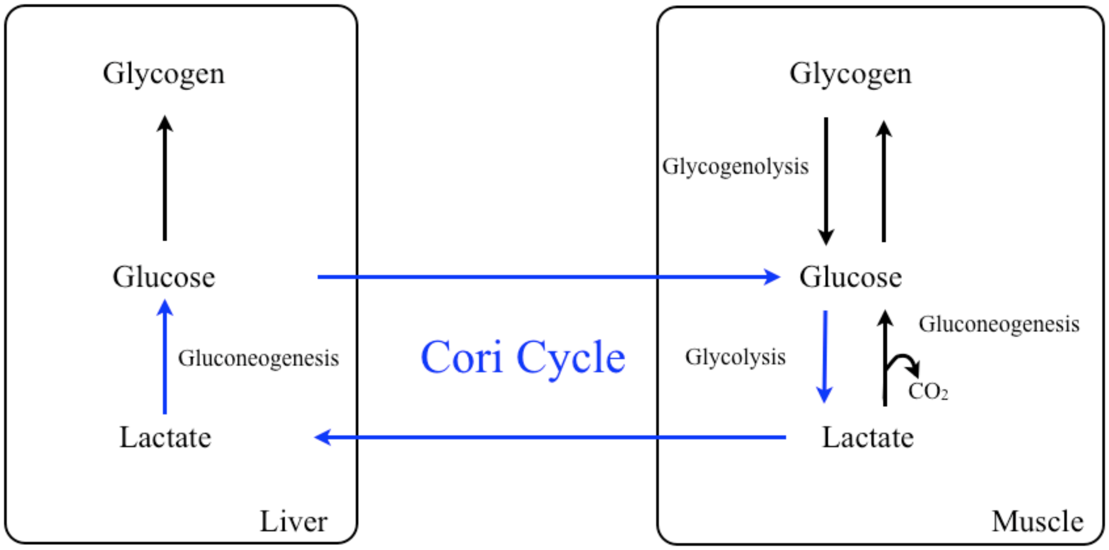

Figure 4. The Cori cycle and lactate metabolism in lizard skeletal muscle as described in Gleeson and DaLession, 1989.

D. Peniche and the Berlengas Archipelago

The Berlengas archipelago is located 5.7 nautical miles from Cape Carvoeiro on the Portuguese coast (Figures 5 and 6). The archipelago is comprised of three sets of islands: the island of Berlenga Grande associated with a few islets, the Estelas islands and the Farilhões-Forcadas islands.

The rocks that form the Berlenga Grande island (herein referred to as Berlenga) and the Farilhões islands appear to be around 270 to 280 million years old. This archipelago is the remnant of an ancient submerged mountain range (Figure 7). Berlenga and the Estelas islands are constituted mainly of granite, whereas the Farilhões-Forcadas islands are formed mainly from ancient metamorphic rock, such as gneisses and gneiss-shales. The island of Farilhão Grande (herein referred to as Farilhão) is assumed to bethe oldest island of the archipelago due to the highest depth of the cliff that separates it from the remaining islands (Almeida, 1993).

The land masses of Peniche and Baleal are made of distinct types of rock from the Berlengas archipelago, mainly limestone, except for a small section in Peniche which contains both granite and gneiss similar to the archipelago (ICBN, 2007).

In the XII century, Peniche and Baleal were islands along the Portuguese coast, only accessible by boat (Blot, ?). However, through the deposition of sand particles, two tombolos were formed, connecting these islands to the mainland around the XVI century (Figure 8) (Calado, 1994).

Figure 5. Map of Portugal with the region of Peniche within the square.

Figure 6. Detailed map of the study region.

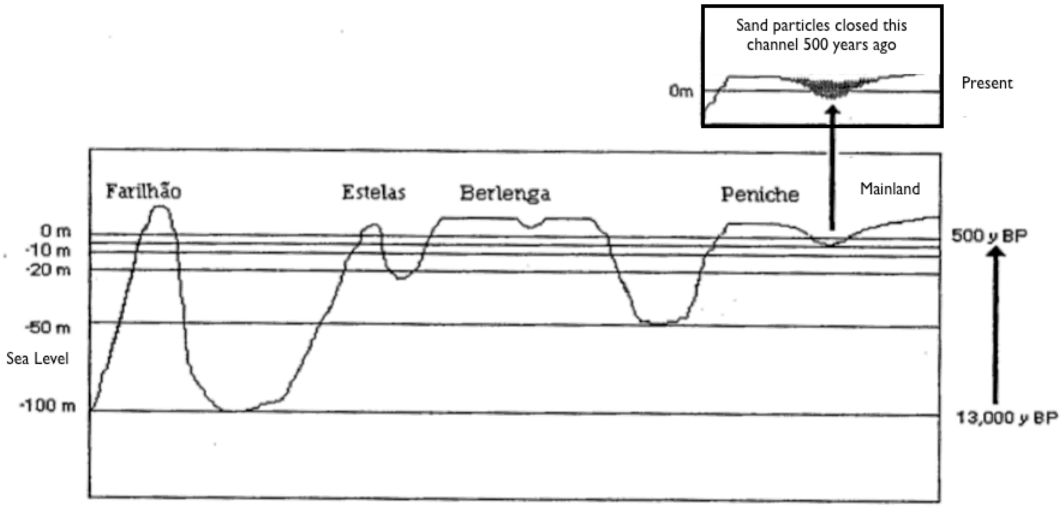

Figure 7. Submerged mountain range that gave rise to the Berlengas archipelago (adapted from Almeida, 1993).

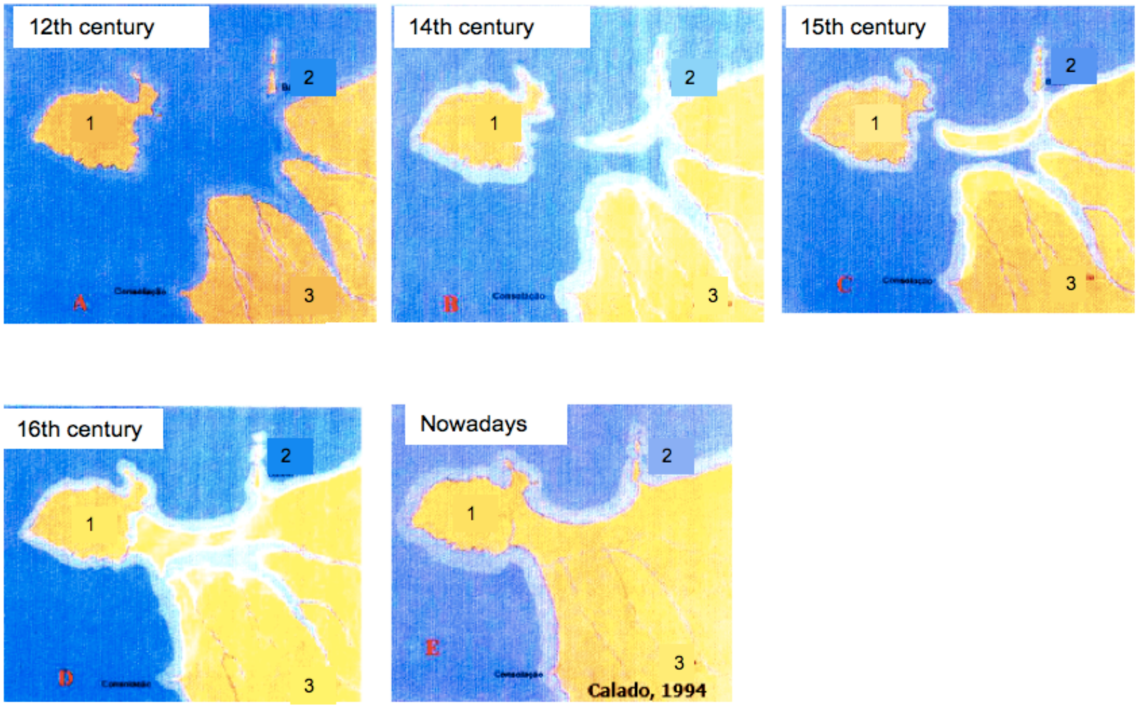

Figure 8. Diagram of the formation of the tombolos of Peniche and Baleal. 1- Peniche , 2- Baleal, 3- Atouguia da Baleia (adapted from Calado, 1994).

E. ***Podarcis carbonelli*** **in the Berlengas archipelago**

In 1985 Vicente described the population present in the Berlengas archipelago as subspecies of *P. bocagei, P.b. berlengensis*. This classification was changed to *P. c. berlengensis* when *P. carbonelli* was acknowledged as a full species (Sá-Sousa and Harris, 2002). However, in 2000 and 2001, two studies using allozymes, biometric data and mtDNA, compared the relationships among the population on the Berlenga Island and several discrete populations of *P. carbonelli* from the mainland, including a population in Peniche, and a high genetic resemblance was found among the populations, hence, they were not considered genetically distinct (Harris and Sá-Sousa, 2001). Nonetheless, the subspecies designation for *P. c. berlengensis* was maintained due to the conspicuous morphological differences between the island and continental forms.

As for the genetic variability of *P. carbonelli* in the archipelago of Berlengas, there is only one study that was performed by the Portuguese Institute for the Conservation of Nature and Biodiversity. In this study, the populations occurring in the Berlengas archipelago were sampled in the island of Berlenga, Farilhão and other islets of the archipelago, as well as Peniche. The study used electrophoresis of isozymes to determine genetic variability of the different populations. The population on the island of Farilhão had the highest levels of heterozygosity of the island populations and was the only island population that had a specific allele, ALB ^{102} , which was shared by all continental populations. These results were somewhat unexpected as a smaller island population should have lower levels of genetic diversity when compared to a mainland population due to the effects of genetic drift that tend to affect smaller populations more drastically (Almeida, 1993).

The *P. carbonelli* lizards found in the island of Berlenga also have several morphologically distinct features from the individuals of the same species found in the mainland. Vicente, in 1989, described a series of morphological and reproductive differences present in these organisms, probably due to the insularity effect. Specifically, *P. carbonelli* on the islands of Berlenga and Farilhão appears to be a giant variant of the species, and the Farilhão population has a higher degree of melanism when compared to the population of Berlenga (Almeida, 1993). The gigantism effect is fairly common in insular reptile species, and *P. carbonelli* on the island of Berlenga appears to be stabilizing as a K-strategist, with higher parental investment (Vicente, 1989). This hypothesis is further supported by observations that lizard egg clutches in Berlenga have double the weight and size of clutches from the mainland. Another well-known effect of insularity is the increase in intraspecific competition associated with agonistic behavior of males, due to a decrease in predatory pressure (Vicente, 1989). It is likely that these behavioral changes, that are a direct consequence of the lizard's unique environment might have a physiological effect as well. If predatory pressure is reduced, the behaviors associated to predator escape will not be observed as often. This reduction in metabolically intense activities, such as shuttling or running from predators, might change the metabolic signature in a way that is detectable across populations.

II. **Objectives**

In this study, population divergence was studied in populations of *P. carbonelli* from the Berlengas archipelago and mainland locations of Peniche and Baleal. Specifically, mitochondrial and nuclear DNA sequences were used to determine whether:

a. The island populations have lower genetic diversity when compared to the mainland populations.

b. The island populations are genetically more distinct than mainland populations due to their isolation.

c. The islands of Berlenga and Farilhão have both ancestral and unique alleles when compared to the mainland populations.

d. The populations of Peniche and Baleal have both ancestral and unique alleles when compared to the islands and to the rest of the peninsula.

e. The mtDNA genetic diversity correlates with nuclear DNA sequence diversity and population structure and divergence scenarios are consistent between molecular markers.

Furthermore, morphometric data were used to determine the extent of documented (in the Berlenga and Peniche populations) and undocumented differentiation in morphology among the four populations studied and divergence in body proportions among the four populations.

Finally, the concentration of blood lactic acid was assayed in the four populations studied to determine whether the reduced predatory pressure in island populations resulted in generally lower blood lactic acid concentrations as a result of less frequent use of anaerobic activity.

III. Materials and methods

A. Sampling

During the summers of 2007 and 2008, individuals of *P. carbonelli* were collected from populations occurring in Berlenga, Farilhão, Peniche and Baleal using a fishing pole to noose them (figure with lizard being noosed). All individuals sampled were weighed with a portable scale, and their snout-vent length (SVL) was recorded. All individuals were marked to avoid resampling.

1. Tissue samples

Tail clippings from *P. carbonelli* were collected from populations sampled from Berlenga, Farilhão, Peniche and Baleal. From these samples, total DNA was isolated using a piece of tissue 0.3 cm long. The tissue was fragmented using a scalpel, and DNA was extracted using the DNAeasy Blood and Tissue kit from Qiagen according to the manufacturer's protocol.

Following DNA isolation, mtDNA sequences corresponding to the control region, 12s rRNA and the 7th nuclear intron of the Beta-fibrinogen gene were amplified by Polymerase Chain Reaction (PCR) using specific primers as indicated in Table 1. The PCR conditions for each primer pair are specified in Table 2.

Each PCR product was run on a 1.3-1.5% agarose gel to confirm the amplicon molecular weight and subsequently cleaned using Promega's Wizard® SV Gel and PCR Clean-Up System. PCR amplicons were sequenced by dideoxysequencing using BigDye® Terminator 3.1 reagents from Applied Biosystems. The fragments were resolved using an Applied Biosystems 3130 sequencer housed in the Western Kentucky University Biotechnology Center and an ABI PRISM 3730xl. The resultant chromatograms were analyzed using the software Sequencher 4.8, and sequences were assembled using CLC Sequence Viewer 6.0.2.

Table 1. Primers used in this study.

Region to Amplify	Primer Sequence	Reference
12S RNA	L1091– 5´- AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT- 3´	Kocher et. al, 1989
	H1478– 5´- TGA CTG CAG AGG GTG ACG GGC GGT GTG T- 3´	
Control Region	DL3F– 5´- GGC CTC TGG TTA ATG GGT TAG TTA C -3´	Crochet et. al, 2004
	Dl4R– 5´- AAT TGT TGG TAG GGG GGT AGG- 3´	
Intron 7 β-fibrinogen	BfibR– 5’-CAG GGA GAG CTA CTT TTG ATT AGA C-3’	Pinho et. al, 2008
	BF8– 5’-CAC CAC CGT CTT CTT TGG AAC ACT G-3’	

Table 2. PCR conditions used for each pair of primers.

Region to Amplify	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
12S RNA	5 min at 94ºC	1 min at 92ºC	1 min at 50ºC	90 s at 72ºC	5 min at 72ºC
Control Region	5 min at 94ºC	1 min at 92ºC	1 min at 50ºC	90 s at 72ºC	5 min at 72ºC
Intron 7 β-fibrinogen	5 min at 94ºC	1 min at 92ºC	1 min at 50-55ºC	90 s at 72ºC	5 min at 72ºC

2. Morphometry

For the morphometric analysis, only adult lizards of *P. carbonelli* were sampled from the populations of Berlenga, Farilhão, Peniche, Baleal and from an isolated population in southern Spain, Doñana. This population was included to provide comparison from a population that is physically distant from our study site.

Lizard gender was determined by observing gender diagnostic features (Sá-Sousa, 2004). To compare the morphometry and physical condition among populations, all individuals sampled were weighed and 7 biometric characters were measured: snout-vent length (SVL), trunk length (TRL), head length from the tip of the snout to the posterior border of the collar (HL), pileus length (PL), head width (HW), front and hind foot length (FFL and HFL respectively), as in Kaliontzopoulou et al. (2007). The biometric characters used in this study pertaining to the head are know to scale isometrically in *P. carbonelli* (Kaliontzopoulou et al. 2007) and the remaining measurements are known to scale isometrically in its sister species *P. hispanica* (Kaliontzopoulou et. al, 2006). Therefore, it was assumed that all biometric characters used scale isometrically in *P. carbonelli*. All measurements were taken from the right side (when applicable), to the closest 0.01 mm using calipers and were performed in the field.

3. Blood lactate concentration

Blood lactate concentration was assayed in the field. The lactate blood levels were obtained using a portable lactate meter from Lactate plus®. The blood samples were obtained by collecting blood that formed naturally after tail clipping. Blood lactate was measured immediately using 0.7 µl of blood.

It has been demonstrated that excessive handling of lizards increases stress levels which in turn increase the concentration of lactate in the blood (Bennett, 1994; Donovan and Gleeson, 2006). As only blood drops that formed immediately after the clipping of the tail were used, this reduced the amount of samples obtainable. Some of the individuals did not bleed immediately after having the tail clipped or did not bleed at all. Therefore, the specimens that did not bleed immediately after the clipping of the tail were not sampled to avoid biasing blood lactate concentration values.

B. Data analysis

1. Molecular data

A total of 86 individuals were analyzed: 17 from Baleal, 20 from Peniche, 24 from Berlenga and 25 from Farilhão. Since only one haplotype was found in all populations for the 12s RNA and control region when sampling 50 individuals equally distributed among the four populations, no further analyses were performed in these two DNA regions.

Because the nuclear intron is the diploid, some individuals were heterozygous in this region. Therefore, the software DnaSP (Librado and Rozas, 2009) was used to reconstruct the haplotypes of heterozygous individuals. The Phase option was used with 1000 iterations and no recombination (Stephens et al. 2001;Stephens and Donelly 2003). DnaSP was used to determine in each population: number of polymorphic sites, number of haplotypes, nucleotide diversity (Nei, 1987, Eq. (10.5)) and haplotype diversity (Nei, 1987, Eqs. (8.4) and (8.12)). Gene differentiation indices, Gst (for haplotype information) and γst (for sequence data information) and corresponding number of migrants (Nm) were also determined in DnaSP using gaps as a 5th state (Nei, 1973 and 1982). Gst, which allows determination of haplotype differentiation between two populations only takes into account if the haplotypes present are different, while γst determines the differentiation between populations based on the nucleotide sequences, accounting for differences in nucleotide in the same position (Lynch and Crease, 1990). Both estimates provided distinct results for inter-population comparisons. The significance of differentiation among all population pairs was assessed using the haplotype -based test statistic Hs,Hst and nucleotide sequence-based statistic Ks*,Kst* by the Permutation method with 1000 replicates (Hudson et al. 1992).

The software Arlequin 3.1 (Excoffier and Schneider, 2005), an Analysis of Molecular Variance (AMOVA) (Excoffier et. al, 1992) tested the following geographic structure: Island (Farilhão and Berlenga) vs. Mainland (Peniche and Baleal). The covariance components obtained were used to determine Φ-statistics at the hierarchical levels defined (within population, among populations within the two groups and among groups), and significance of the variance components and Φ-statistics was determined through a non-parametric permutation approach in Arlequin with 1023 permutations.

One concern regarding the use of nuclear genes for phylogeographic questions is the possible occurrence of recombination, particularly at rates similar to nucleotide substitution at a locus (Hare, 2001). This situation might introduce homoplasy into the data set and give rise to erroneous results. To determine whether recombinant sequences were present in my data set, the sequences were analyzed with RDP v3.34 (Martin et. al, 2005a), a software that detects recombinant sequences and recombination break points using the following automatic methods: rdp (Martin and Rybicki, 2000), geneconv (Padidam et. al, 1999), maximum χ (Maynard Smith, 1992), bootscan (Martin et. al, 2005b), chimaera (Posada and Crandall, 2001) and sister scanning (Gibbs et. al, 2000).

To determine which of the haplotypes found were exclusive of island populations, and exclusive of Peniche and Baleal, I aligned the alleles found in this study with sequences for the same nuclear region deposited in GenBank for this species (sequences accession numbers EU269475 up to EU269481 from Pinho et. al, 2008). Through observation, I determined which haplotypes found in this study were not found in remaining mainland populations of *P. carbonelli*.

A haplotype network was built using the software TCS (Clement et. al, 2000) with a 95% probability of parsimoniuos connection, and the program Network (www.fluxus-engineering.com), to represent the relative fraction of each haplotype in each population. Network ambiguities were resolved following the rules in Pfenninger and Posada (2000). The haplotypes were grouped into hierarchical clades, and significance of association between geographic and genetic distances of clades was determined in GeoDis (Posada et. al, 2000) with 10,000 permutations. The population processes underlying the observed clade distribution were determined for the clades with significant geographical associations using the GeoDis inference key, based on Templeton, 1998. All statistical software used is freely available on the Internet.

2. Morphometry

Female measurements and male measurements were treated separately due to the fact that morphometric sexual dimorphism occurs in this species (Sá-Sousa, 2004). The variable “body condition” was calculated for all individuals sampled by dividing the body mass by the snout-vent length (SVL). Body condition, along with body mass and SVL were tested for normality in all populations and both genders, with a Shapiro-Wilk normality test, and for homocedasticity, with a Levenne’s equality of several variances test. Data were not normal nor homoscedastic, and no data transformation was found that improved normality and homoscedasticity. A Kruskal-Wallis test, followed by a Dwass-Steel-Chritchlow-Fligner all pairwise comparisons test, was performed in both males and females, separately, for each variable, to test for differences among populations.

To determine whether the absolute body measurements and body proportions (HL/SVL, TRL/SVL, FFL/SVL, HFL/SVL, PL/SVL, HW/SVL) were diagnostic of each population, two canonical discriminant analyses were performed using the four populations of this study (Berlenga, Baleal, Farilhão and Peniche), and the population of Doñana.

The body proportions were tested for normality for each population and homogeneity of variances between populations. In both genders, the majority of variables was normal and all were homoscedastic, except for the variable PL/SVL which was not homoscedastic in males.

Averages of the canonical scores for each population, for both absolute and relative measurements, were clustered using complete and single linkage to determine similarity among populations. The canonical scores of the canonical analysis were used to perform the cluster analysis, because they provide a set of uncorrelated data points, and these can be clustered using Euclidian distances. Furthermore, the canonical scores serve as coordinates of the canonical axes, which means that the average of a population's scores will represent the average position, in canonical space, of the that population. The reasoning behind choosing to average the canonical scores was due to lack of segregation by the samples in the cluster tree and the high number of samples in the analysis. The resulting cluster tree did not group all the individuals of a same population together, which when associated with a high number of samples further complicates interpretation of the results. Therefore, as the interest of this analysis was to obtain an overall measurement of similarity, the population averages of the canonical scores were clustered instead.

As the ANOVA is robust enough to tolerate deviations for normality, one-way ANOVAs were performed to determine whether there were significant differences in the several body proportions among populations. When necessary, a Tukey HSD Multiple Comparisons Test was performed to determine which populations were significantly different. For the variable PL/SVL in males a Kruskal-Wallis test was performed. The α used in these analyses was 0.05. Kruskal-Wallis post-hoc tests were performed in StatsDirect® while all remaining tests were performed in Systat®.

3. Lactate assay data

Blood lactate concentrations (mmol/l) were tested for normality with a Shapiro-Wilk normality test and homocedasticity with a Levenne’s test for all groups. The majority of the groups were normal (Shapiro-Wilk P-Value >0.05) and homoscedastic (Levenne’s test p-value >0.05). A multifactorial ANOVA was performed on the data set to determine the effects of gender and location on blood lactate levels, followed by a Tukey post hoc test. The α used in these analyses was 0.05 and all data were analyzed in STATISTICA®.

IV. **Results**

A. Molecular Data

The sequence obtained for the β-fibrinogen intron-7 was 636 bp long. This fragment was manually trimmed to a 586 bp sequence successfully amplified for all samples. A total of 25 polymorphic sites were found and a 16 bp deletion was also found in two of the samples at position 136. This deletion was considered as a single event (instead of 16 events) in the analyses. A total of 22 distinct haplotypes were found (Table 3 and 4).

Total haplotype diversity was 0.709 and total nucleotide diversity was 0.01073. Haplotype and nucleotide diversity was highest in Peniche. In the island populations, Berlenga has a higher haplotype diversity than Farilhão although the latter had a higher nucleotide diversity than Berlenga (Table 5).

The haplotype-based estimate presented the populations of Berlenga and Peniche as the most different (Gst= 0.08969), and consequently with the lower number of migrants (Nm=2.54), while the populations of Farilhão and Berlenga were the most similar (Gst=0.02303), with higher number of migrants (Nm=10.61) (Table 6 and 7).

Table 3. Haplotypes found for the β-fibrinogen intron 7. "." represents a base identical to the header of the table; "-" represents a 16 bp deletion; "?" represents an unknown base.

	29	61	77	90	121	142	164	165	183	196	201	202	209	212	215	217	219	240	262	285	296	297	317	348	368	585
Hap_1	T	G	G	G	T	C	C	A	T	C	T	G	A	A	A	G	G	G	C	G	C	A	A	C	T	G
Hap_2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
Hap_3	.	.	.	.	.	.	.	G	C	T	C	A	G	G	G	T	T	C	A	.	.	G	G	T	.	.
Hap_4	.	.	.	.	.	.	.	G	C	T	C	A	G	G	G	T	T	C	A	.	.	G	G	.	.	C
Hap_5	.	.	.	.	.	.	.	G	C	T	C	A	G	G	G	T	T	C	A	.	.	G	G	.	.	.
Hap_6	.	.	A	.	.	.	.	G	C	T	C	A	G	G	G	T	T	.	A	.	.	G	G	.	.	C
Hap_7	.	A	.	A	.	.	T	G	C	G	C	A	G	G	.	A	C	.	A	.	T	G	G	.	.	.
Hap_8	C	.	.	.	–	.	.	G	C	G	C	A	G	G	.	A	T	.	A	A	.	G	G	.	.	.
Hap_9	.	.	.	.	.	.	.	G	C	G	C	A	G	G	.	A	T	.	A	A	.	G	G	.	.	.
Hap_10	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	G	G	.	.	.
Hap_11	.	A	.	.	.	T	.	G	C	G	C	A	G	G	G	A	C	.	A	.	T	G	G	.	.	.
Hap_12	.	.	A	.	.	.	.	G	C	T	C	A	G	G	G	T	T	.	A	.	.	G	G	.	.	.
Hap_13	.	.	A	.	.	.	.	G	C	T	C	A	G	G	G	T	T	C	A	.	.	G	G	.	.	C
Hap_14	.	.	.	.	.	.	.	G	C	T	C	A	G	G	G	T	T	C	A	.	.	G	G	.	C	C
Hap_15	.	.	A	.	.	.	.	G	C	T	C	A	G	G	G	T	T	.	A	.	.	G	G	.	C	C
Hap_16	.	.	.	.	.	.	.	.	C	T	C	A	G	G	G	T	T	A	A	.	.	G	G	.	.	C
Hap_17	.	.	.	.	.	.	.	G	C	G	C	A	G	G	G	T	T	C	A	.	.	G	G	.	.	.
Hap_18	.	.	.	.	.	.	.	G	C	T	C	A	G	G	.	A	T	.	A	.	.	G	G	.	.	.
Hap_19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	G	.	.	C
Hap_20	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.
Hap_21	C	.	.	?	.	.	.	G	C	G	C	A	G	G	.	A	T	.	A	A	.	G	G	.	.	.
Hap_22	C	.	.	?	.	.	.	G	C	T	C	A	G	G	G	T	T	C	A	.	.	G	G	.	.	C

Table 4. Number of chromosomes in which each haplotype was found, by population.

	Berlenga	Farilhão	Peniche	Baleal	Total
Hap_1	0	3	14	5	22
Hap_2	0	7	0	0	7
Hap_3	0	0	0	4	4
Hap_4	27	33	12	16	88
Hap_5	2	1	0	0	3
Hap_6	0	0	6	2	8
Hap_7	0	0	3	0	3
Hap_8	4	0	0	0	4
Hap_9	13	4	1	0	18
Hap_10	0	0	1	0	1
Hap_11	0	0	1	0	1
Hap_12	0	0	1	0	1
Hap_13	0	0	0	1	1
Hap_14	0	0	0	1	1
Hap_15	0	0	0	1	1
Hap_16	0	1	0	0	1
Hap_17	0	0	2	0	2
Hap_18	0	0	0	1	1
Hap_19	0	0	0	2	2
Hap_20	0	1	0	0	1
Hap_21	1	0	0	0	1
Hap_22	1	0	0	0	1

Table 5. Parameters estimated from the β-fibrinogen intron-7 of *Podarcis carbonelli*.

Population	**N**	**Number of Haplotypes**	**Haplotype diversity**	**Nucleotide diversity**	**Polymorphic sites**
Baleal	17	9	0.725	0.00963	18
Peniche	20	9	0.788	0.01472	22
Berlenga	24	6	0.613	0.00531	7
Farilhão	25	7	0.544	0.01047	17

Table 6. Gene differentiation index Gst (Nei, 1973) between the populations *Podarcis carbonelli.*

Population	**Baleal**	**Peniche**	**Berlenga**	**Farilhão**
Baleal				
Peniche	0.02881*			
Berlenga	0.03531*	0.08969*		
Farilhão	0.02045*	0.08902*	0.02303*	

Note: The pairs of populations that are statistically significant from 0 ($p<0.05$) according to a PM test, with 1000 replicates, for Hs, Hst are followed by *.

Table 7. Number of migrants (Nm) associated with Gst (Nei, 1973).

Population	**Baleal**	**Peniche**	**Berlenga**	**Farilhão**
Baleal				
Peniche	8.43			
Berlenga	6.83	2.54		
Farilhão	11.98	2.56	10.61	

The estimate based on the nucleotide sequences also showed Peniche and Berlenga as the most distinct populations (γst=0.14767) with the populations of Baleal and Farilhão being the most similar (γst=0.00672) with an extremely high number of migrants (Nm=36.95) (Tables 8 and 9).

Table 8. Gene differentiation index γst (Nei, 1982) between the populations *Podarcis carbonelli*.

Population	Baleal	Peniche	Berlenga	Farilhão
Baleal				
Peniche	0.0532*			
Berlenga	0.0846*	0.1477*		
Farilhão	0.0067	0.0428*	0.0753*	

Note: The pairs of populations that are statistically significant from 0 ($p<0.05$) according to a PM test, with 1000 replicates, for Ks*, Kst* are followed by *.

Table 9. Number of migrants associated with γst (Nei, 1982) between *Podarcis carbonelli* populations.

Population	Baleal	Peniche	Berlenga	Farilhão
Baleal				
Peniche	4.45			
Berlenga	2.7	1.44		
Farilhão	36.95	5.59	3.07	

The AMOVA showed that the majority of genetic variation is found within populations. Furthermore, the genetic structure found among the island region and mainland is not statistically significant but there is a statistically significant low genetic structure among all populations and among populations within groups (Table 10).

Table 10. Results for the AMOVA. Significance of the fixation indices (when $p<0.05$) is indicated with an *.

Source of variation	**d.f.**	**Sum of Squares**	**Percentage of variation**	**Φ - Statistics**
Among groups	1	3.342	7.22	$\Phi_{CT}=0.07223$
Among populations within groups	2	2.166	4.73	$\Phi_{SC}=0.05096$*
Within populations	168	55.102	88.05	$\Phi_{ST}=0.11951$*

The software RDP did not reveal any recombinant sequences when using all 6 automatic methods :rdp, geneconv, maximum χ , bootscan, chimaera and sister scanning. The alignment between the haplotypes found in this study and the haplotypes that had been previously described for this species identified a match of two haplotypes: the haplotype with the entry number EU269477 was Hap_1 from this study, and EU269479 was Hap_5 from this study. No other haplotypes were identified that matched the information in this data set.

The haplotype network built based on Templeton, 1998, showed a lack of genetic structure; a few haplotypes were exclusive of one of the populations, but these occurred only in single individuals (Figure 9) . TCS identified Haplotype 5 (H_5) as the most likely outgroup for this network (outgroup weight= 0.243). The nesting of the network resulted into 14 informative nested clades (Figure 10).

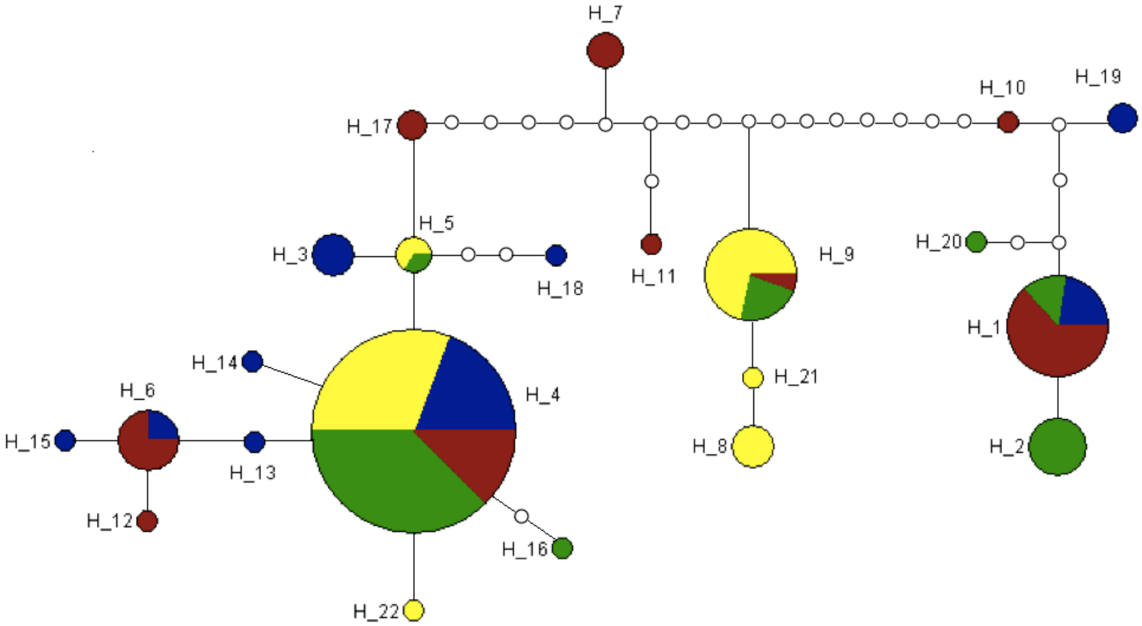

Figure 9. The haplotype networks estimated by the method described in Templeton, 1998, for the 7th intron of the β-fibrinogen gene. Haplotype labels are by each node. When two nodes are connected directly, only one change has occurred from one haplotype to the other. White circles represent missing haplotypes identified by parsimony. The size of the circles is proportional to the number of individuals that have that haplotype. Color code: Green: population of Farilhão; Blue: population of Baleal; Yellow: population of Berlenga; Red: population of Peniche.

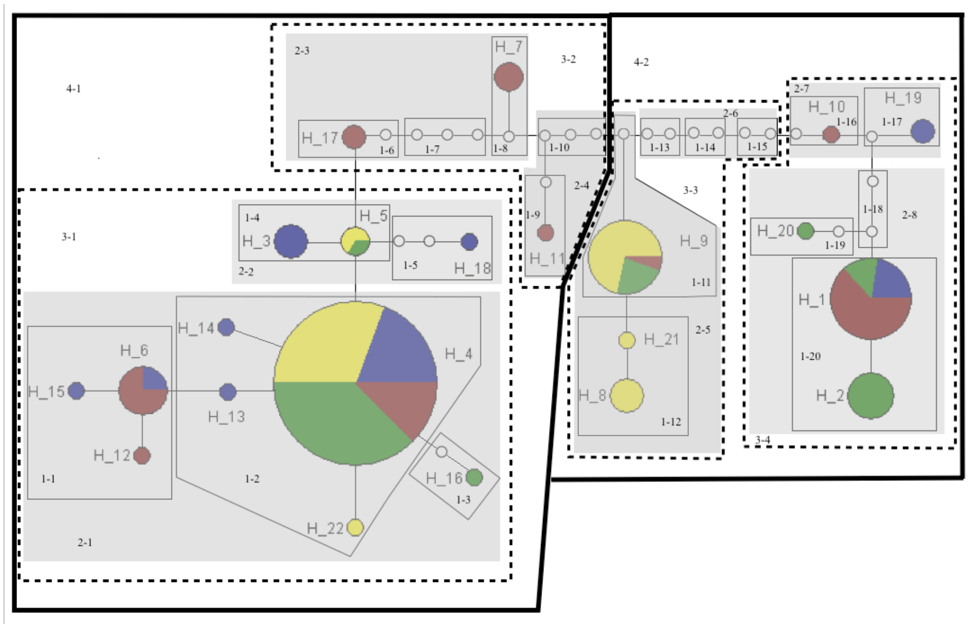

Figure 10. Haplotype network with nested clades. Clades nested within thin line shapes are step-1; clades nested within grey boxes are step-2 clades; clades nested within dashed-lines are step-3 clades and clades nested within thick lines are step-4 clades. Haplotype labels are by each node. When two nodes are connected directly, only one change has occurred from one haplotype to the other. White circles represent missing haplotypes identified by parsimony. The size of the circles is proportional to the number of individuals that have that haplotype.

The total cladogram did not show a significant association between geographical distribution and genetic composition of populations. However, some of the lower clades did show significant results. Specifically, clades 1-20, 2-1, 4-1 and 4-2 show a statistically significant association haplotype distribution and population distribution; the common trend among these clades is that the current association between geography and genetic composition of the clades is due to restricted gene flow (see Table 11).

Table 11. Biogeographical inferences for nested clades of *Podarcis carbonelli* with significant phylogeographic structure.

Clade	**Inference key steps**	**Inferred historic event**
1-20	2-11-17-4	Restricted Gene Flow with Isolation by Distance
2-1	2-11-12	Contiguous Range Expansion
4-1	2-3-4	Restricted Gene Flow with Isolation by Distance
4-2	2-3-4	Restricted Gene Flow with Isolation by Distance

B. Morphometric data

1. <u>One-Way Anova with SVL, Mass and Body condition</u>

A total of 230 individuals were measured. Of these 140 were males (Farilhão=49, Peniche=22, Berlenga=49, Baleal=11, Doñana=9) and 90 were females (Farilhão=37, Peniche=12, Berlenga=27, Baleal=7, Doñana=7). Table 12 contains the population averages, by gender, for all morphological variables measured.

Samples were not normal and there was no homogeneity of variances. Consequently, the Kruskal-Wallis ANOVA test, was used to determine whether there were any significant differences in body mass, SVL and body condition among populations, in both sexes.

Significant differences were found among female populations for mass (Kruskal-Wallis Test Statistic = 57.255, $p < 0.001$, df= 4), SVL (Kruskal-Wallis Test Statistic = 48.030, $p < 0.001$, df=4) and body condition (Kruskal-Wallis Test Statistic = 56.086, p <

0.001, df= 4). Females from Farilhão had significantly higher body mass, SVL and body condition compared to females from other populations (see Tables 13,14,15 and Figures 11 and 12).

Table 12. Population means for the morphological variables measured.

	Berlenga	**Farilhão**	**Peniche**	**Baleal**	**Doñana**
Mass males	5.24	7.90	3.88	4.68	2.63
SVL males	5.91	6.78	5.50	5.85	5.05
HL males	2.12	2.49	1.97	2.19	1.82
HW males	0.80	0.98	0.72	0.77	0.71
TRL males	3.56	4.06	3.40	4.13	2.62
FFL males	1.87	2.22	1.73	1.96	1.72
HFL males	2.91	3.39	2.71	2.64	2.75
PL males	1.41	1.67	1.41	1.51	1.22
Mass females	3.39	4.78	2.71	2.73	2.13
SVL females	5.55	6.17	5.34	5.27	4.95
HL females	1.76	2.01	1.79	1.74	1.59
HW females	0.65	0.72	0.58	0.57	0.59
TRL females	3.62	3.89	3.41	3.57	2.65
FFL females	1.5	1.84	1.54	1.51	1.44
HFL females	2.37	2.75	2.31	2.16	2.27
PL females	1.17	1.32	1.12	1.08	1.04

Table 13. Results of the a Dwass-Steel-Chritchlow-Fligner all pairwise comparisons test for the variable mass in females. * indicates a statistically significant result.

	Baleal	Peniche	Berlenga	Farilhão	Doñana
Baleal					
Peniche	p=0.952				
Berlenga	p=0.157	p=0.0017*			
Farilhão	p=0.0047*	p<0.0001*	p<0.0001*		
Doñana	p=0.741	p=0.111	p=0.0024*	p=0.0004*	

Table 14. Results of the a Dwass-Steel-Chritchlow-Fligner all pairwise comparisons test for the variable SVL in females. * indicates a statistically significant result.

	Baleal	Peniche	Berlenga	Farilhão	Doñana
Baleal					
Peniche	p=0.999				
Berlenga	p=0.3796	p=0.292			
Farilhão	p=0.004*	p=0.0002*	p<0.0001*		
Doñana	p=0.499	p=0.125	p=0.014*	p=0.0006*	

Table 15. Results of the a Dwass-Steel-Chritchlow-Fligner all pairwise comparisons test for the variable body condition in females. * indicates a statistically significant result.

	Baleal	Peniche	Berlenga	Farilhão	Doñana
Baleal					
Peniche	p=0.916				
Berlenga	p=0.113	p=0.0024*			
Farilhão	p=0.009*	p<0.0001*	p<0.0001*		
Doñana	p=0.813	p=0.151	p=0.0018*	p=0.0004*	

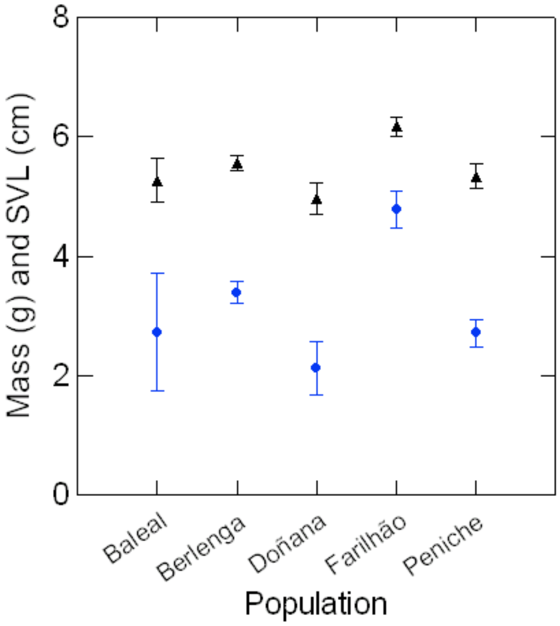

Figure 11. Means of female body mass (g),●, and SVL (cm), ▲, by population. Vertical bars represent the 95% confidence interval of the mean.

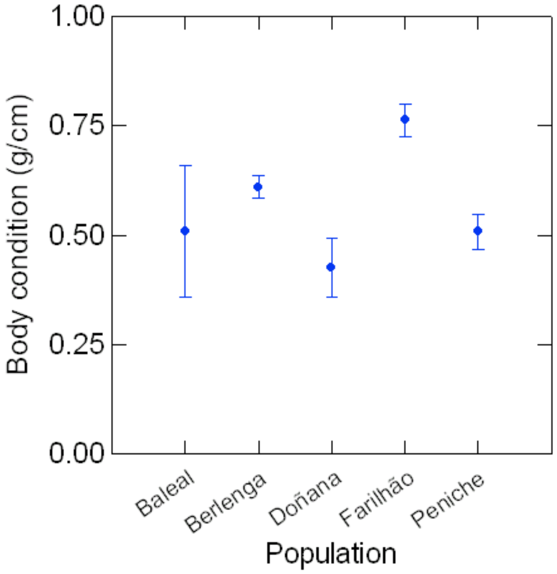

Figure 12. Means of female body condition (g/cm),●, by population. Vertical bars represent the 95% confidence interval of the mean.

Males from the 5 populations had significant differences in body mass (Kruskal-Wallis Test Statistic = 93.118, $p < 0.001$, df= 4), SVL (Kruskal-Wallis Test Statistic = 91.442, $p< 0.001$, df= 4) and body condition (Kruskal-Wallis Test Statistic = 88.374, $p<0.001$, df= 4). The males from Farilhão had significantly higher body mass, SVL and body condition when compared to males from all other populations (see tables 16,17,18 and Figures 13,14).

Table 16. Results of the a Dwass-Steel-Chritchlow-Fligner all pairwise comparisons test for the variable mass in males. * indicates a statistically significant result.

	Baleal	Peniche	Berlenga	Farilhão	Doñana
Baleal					
Peniche	p=0.398				
Berlenga	p=0.661	p<0.0001*			
Farilhão	p<0.0001*	p<0.0001*	p<0.0001*		
Doñana	p=0.0028*	p=0.0018*	p<0.0001*	p<0.0001*	

Table 17. Results of the a Dwass-Steel-Chritchlow-Fligner all pairwise comparisons test for the variable SVL in males. * indicates a statistically significant result.

	Baleal	Peniche	Berlenga	Farilhão	Doñana
Baleal					
Peniche	p=0.470				
Berlenga	p>0.999	p<0.0001*			
Farilhão	p=0.0003*	p<0.0001*	p<0.0001*		
Doñana	p=0.0123*	p=0.0045*	p<0.0001*	p<0.0001*	

Table 18. Results of the a Dwass-Steel-Chritchlow-Fligner all pairwise comparisons test for the variable body condition in Males. * indicates a statistically significant result.

	Baleal	Peniche	Berlenga	Farilhão	Doñana
Baleal					
Peniche	p=0.594				
Berlenga	p=0.3484	p<0.0001*			
Farilhão	p<0.0001*	p<0.0001*	p<0.0001*		
Doñana	p=0.0028*	p=0.0045*	p<0.0001*	p<0.0001*	

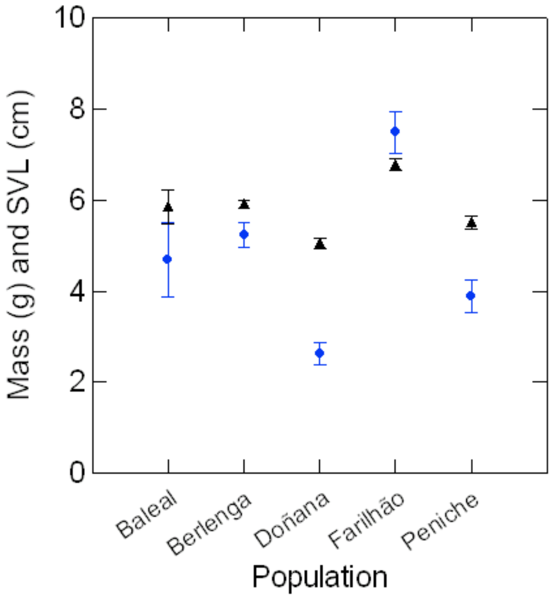

Figure 13. Means of male body mass (g), ●, and SVL (cm), ▲, by population. Vertical bars represent the 95% confidence interval of the mean.

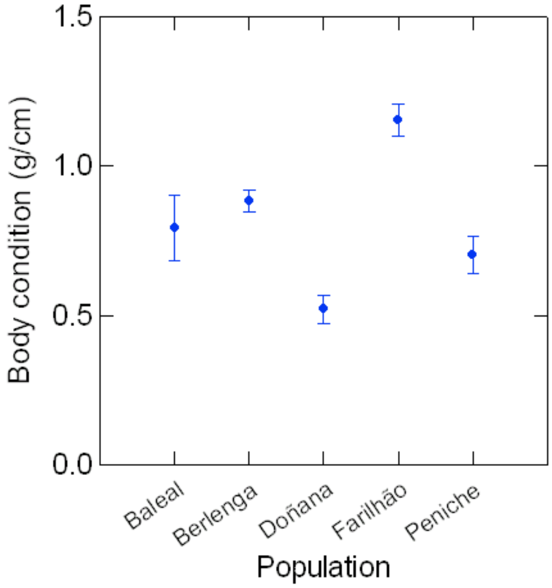

Figure 14. Plot of means of male body condition (g/cm) by population. Bars represent the 95% confidence interval of the mean.

2. Canonical variates analysis

A total of 106 individuals were sampled for 6 biometric characters: TRL, HL, HW, PL, FFL and HFL. From these, 59 were males (Farilhão=20, Peniche=16, Berlenga=10, Baleal=4, Doñana=9) and 47 females (Farilhão=19, Peniche=10, Berlenga=9, Baleal=2, Doñana=7).

The canonical variates analysis using female absolute body measurements as predictors showed significant differences in morphometry among groups (Wilks' lambda= 0.047, $F_{24,130}$= 7.59, p<0.001). All populations were significantly different from each other, except Baleal which was not significantly different from Peniche (Table 19). Classification of the different samples into the correct populations, the overall classification success was 87% (see Table 20).

Table 19. Female absolute measurements between groups *F*-matrix. $F_{0.05,6,37}$=2.36. Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	0.000				
Berlenga	2.392*	0.000			
Doñana	4.861*	11.509*	0.000		
Farilhão	3.422*	7.585*	29.173*	0.000	
Peniche	0.249	7.280*	10.755*	12.252*	0.000

Table 20. Female canonical variates with absolute measurements classification matrix. Each row shows where the individuals of the population were classified.

	Baleal	Berlenga	Doñana	Farilhão	Peniche	%correct
Baleal	0	0	0	0	2	0
Berlenga	0	8	0	0	1	89
Doñana	0	0	6	0	1	86
Farilhão	0	1	0	18	0	95
Peniche	0	0	0	1	9	90
Total	0	9	6	19	13	87

The canonical variates analysis using female body proportions as predictors showed significant differences in morphometry among groups (Wilks' lambda= 0.1195, $F_{24,130}$= 4.5429, p<0.001). All populations were significantly different from the population of Doñana and Farilhão and Peniche were not significantly different from Baleal (Table 21). Classification of the different samples into the correct populations was 81% (see Table 22).

Table 21. Female body proportions between groups *F*-matrix. $F_{0.05,6,37}$=2.36. Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	0.000				
Berlenga	2.432*	0.000			
Doñana	4.474*	8.082*	0.000		
Farilhão	1.261	2.858*	8.565*	0.000	
Peniche	0.570	8.336*	7.010*	5.692*	0.000

Table 22. Female body proportions classification matrix. Each row shows where the individuals of the population were classified.

	Baleal	Berlenga	Doñana	Farilhão	Peniche	%correct
Baleal	0	0	0	1	1	0
Berlenga	0	7	0	2	0	78
Doñana	0	0	7	0	0	100
Farilhão	0	2	0	17	0	89
Peniche	0	0	0	3	7	70
Total	0	9	7	23	8	81

The canonical variates analysis using male absolute body measurements as predictors showed significant differences in morphometry among groups (Wilks' lambda= 0.0663 , $F_{24,172}$= 8.4359, p<0.001). All populations were significantly different from each other (Table 23). Classification of the different samples into the correct populations was 86% (see Table 24).

Table 23. Between groups F-matrix. $F_{0.05,6,49}$=2.29. Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	0.000				
Berlenga	2.775*	0.000			
Doñana	11.381*	6.985*	0.000		
Farilhão	6.361*	9.543*	28.634*	0.000	
Peniche	3.593*	2.689*	8.545*	26.981*	0.000

Table 24. Male absolute measurements classification matrix. Each row shows where the individuals of the population were classified.

	Baleal	Berlenga	Doñana	Farilhão	Peniche	%correct
Baleal	4	0	0	0	0	100
Berlenga	0	9	0	0	1	90
Doñana	0	0	8	0	1	89
Farilhão	0	3	0	17	0	85
Peniche	1	1	1	0	13	81
Total	5	13	9	17	15	86

The canonical variates analysis using male body proportions as predictors showed significant differences in morphometry among groups (Wilks' lambda= 0.2588, $F_{24,172}$= 3.3919, p<0.001). All populations were significantly different from the population of Doñana (Table 25). Classification of the different samples into the correct populations, the overall classification success was 68% (see Table 26).

Table 25. Between groups *F*-matrix. $F_{0.05,6,49}$=2.29. Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	0.000				
Berlenga	2.334*	0.000			
Doñana	8.237*	4.609*	0.000		
Farilhão	3.639*	0.677	4.430*	0.000	
Peniche	1.725	1.760	9.358*	5.178*	0.000

Table 26. Male body proportions classification matrix. Each row shows where the individuals of the population were classified.

	Baleal	Berlenga	Doñana	Farilhão	Peniche	%correct
Baleal	3	0	0	0	1	75
Berlenga	0	0	1	6	3	0
Doñana	0	0	7	1	1	78
Farilhão	0	1	1	15	3	75
Peniche	1	0	1	3	11	69
Total	4	1	10	25	19	61

3. Cluster Analysis

The cluster analysis was performed to determine the overall similarity among populations using the population average scores from the canonical variates analysis with absolute body measurements (HL, HW, TRL, FFL, HFL, PL) and with relative body measurements (HL/SVL, HW/SVL, TRL/SVL, FFl/SVL, HFL/SVL, PL/SVL).

In females, the cluster analyses performed using the canonical scores derived from absolute and relative measurements determined the populations of Peniche and Baleal to be the most similar in terms of size, followed by the populations of Berlenga and Farilhão (Figures 15, 16, 17 and 18).

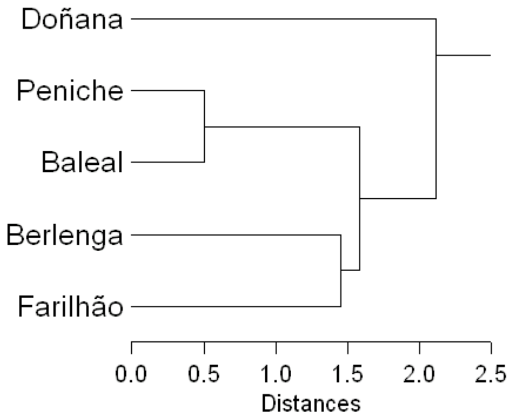

Figure 15. Cluster tree of average canonical scores from female absolute body measurements. The distance used is the Euclidean distance and the clustering method is the Single linkage method (nearest neighbor).

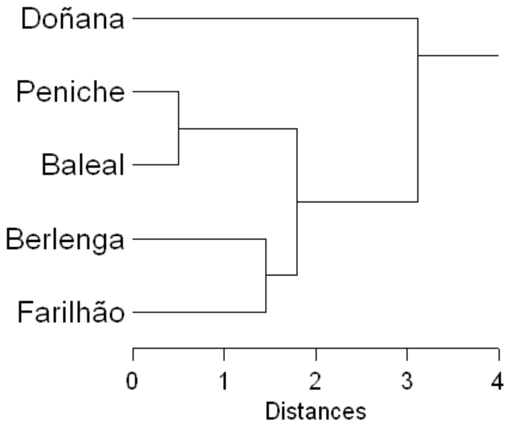

Figure 16. Cluster tree of average canonical scores from female absolute body measurements. The distance used is the Euclidean distance and the clustering method is the Complete linkage method (farthest neighbor).

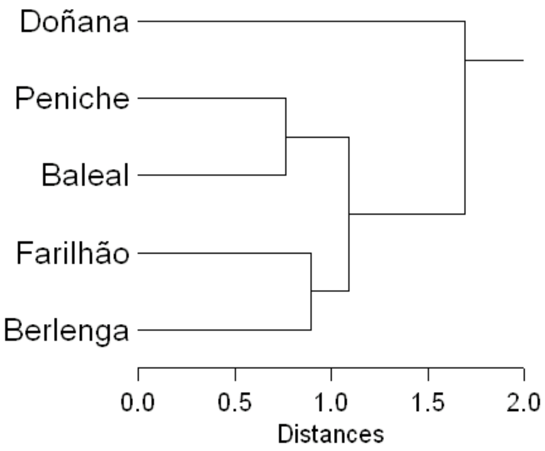

Figure 17. Cluster tree of average canonical scores from female relative body measurements. The distance used is the Euclidean distance and the clustering method is the Single linkage method (nearest neighbor).

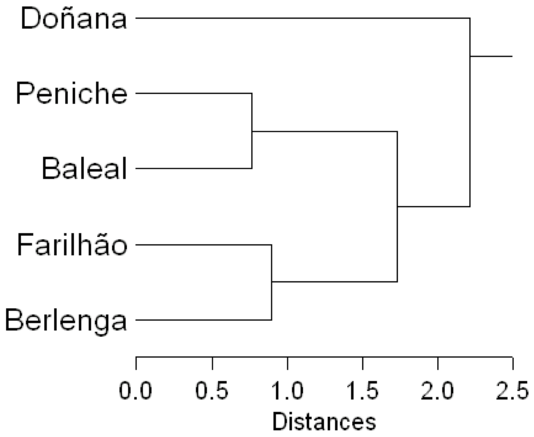

Figure 18. Cluster tree of average canonical scores from female relative body measurements. The distance used is the Euclidean distance and the clustering method is the Complete linkage method (farthest neighbor).

The cluster analyses for males revealed a different trend from the female analyses. The clustering of the average canonical scores from absolute body measurements, both the single and complete linkage methods, clustered the populations of Berlenga and Peniche as the most similar, followed by Baleal and then Farilhão (Figures 19 and 20). As for the clustering with relative body measurements, the populations of Berlenga and Farilhão were, in both methods, clustered together as the most similar (Figures 21 and 22).

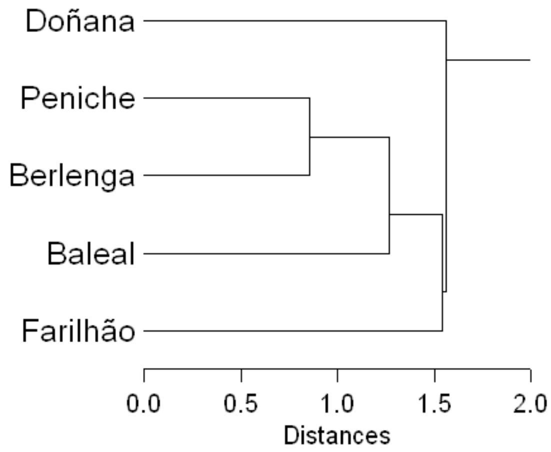

Figure 19. Cluster tree of average canonical scores from male absolute body measurements. The distance used is the Euclidean distance and the clustering method is the Single linkage method (nearest neighbor).

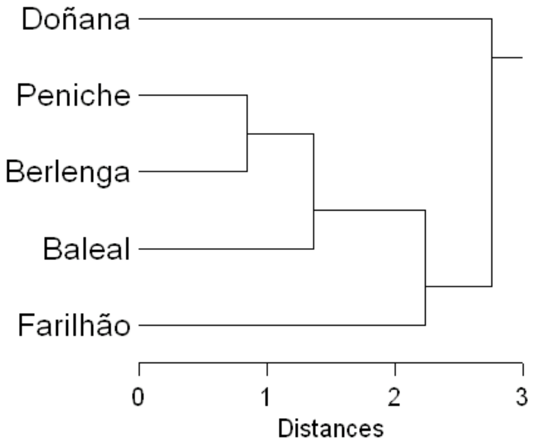

Figure 20. Cluster tree of average canonical scores from male absolute body measurements. The distance used is the Euclidean distance and the clustering method is the Complete linkage method (farthest neighbor).

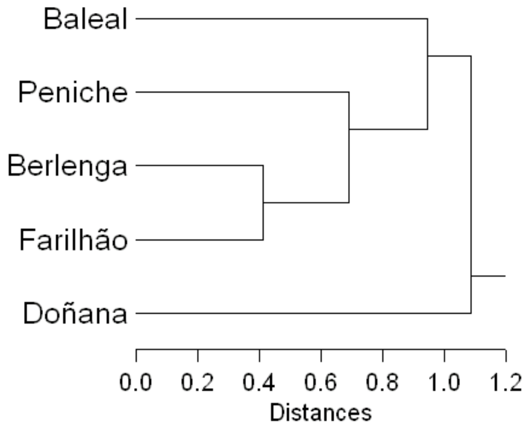

Figure 21. Cluster tree of average canonical scores from male relative body measurements. The distance used is the Euclidean distance and the clustering method is the Single linkage method (nearest neighbor).

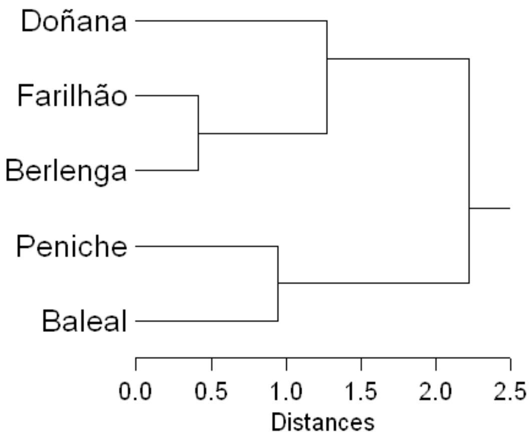

Figure 22. Cluster tree of average canonical scores from male relative body measurements. The distance used is the Euclidean distance and the clustering method is the Complete linkage method (farthest neighbor).

4. One-Way Anova with body proportions

In females only relative head width (HW/SVL), relative trunk length (TRL/SVL) and relative front foot length (FFL/SVL) were significantly different among populations (Table 27).

Table 27. Analysis of variance for 6 standardized morphometric variables among females. Significant results are marked with an *.

Variable	F-ratio	p
HL/SVL	1.610	0.189
HW/SVL	5.141	0.002*
TRL/SVL	15.845	<0.001*
FFL/SVL	3.046	0.027*
HFL/SVL	1.223	0.316
PL/SVL	1.715	0.165

Note: Degrees of freedom for all tests are 4, 42.

The population of Peniche had a significantly narrower head width than the populations of Farilhão and Doñana (see Table 28 and Figure 23). The females in population of Donãna had a significantly shorter relative trunk length than all other populations (Table 29 and Figure 24). Also, females from Berlenga had significantly shorter relative front foot lengths when compared to the population of Farilhão (Table 30, Figure 25).

Table 28. Tukey HSD matrix of pairwise comparison probabilities for female relative head width (HW/SVL). Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	1.000				
Berlenga	0.483	1.000			
Doñana	0.289	0.970	1.000		
Farilhão	0.223	0.938	1.000	1.000	
Peniche	1.000	0.065	0.021*	0.002*	1.000

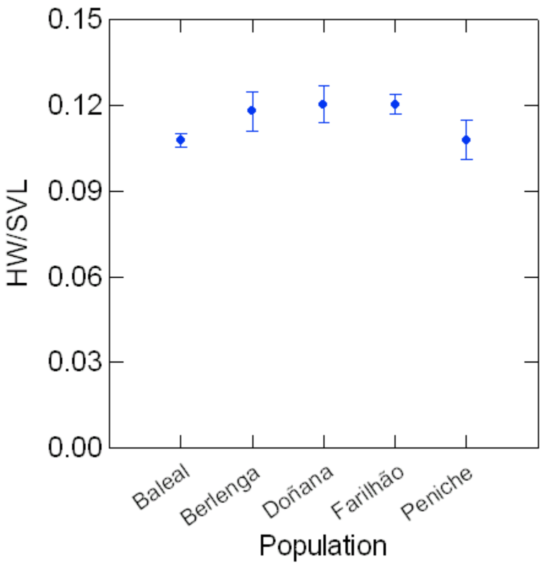

Figure 23. Plot of mean female relative head width (HW/SVL) by population. Bars represent the 95% confidence interval of the mean.

Table 29. Tukey HSD matrix of pairwise comparison probabilities for female relative trunk length (TRL/SVL). Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	1.000				
Berlenga	0.905	1.000			
Doñana	<0.001*	<0.001*	1.000		
Farilhão	0.758	0.988	<0.001*	1.000	
Peniche	0.443	0.641	<0.001*	0.797	1.000

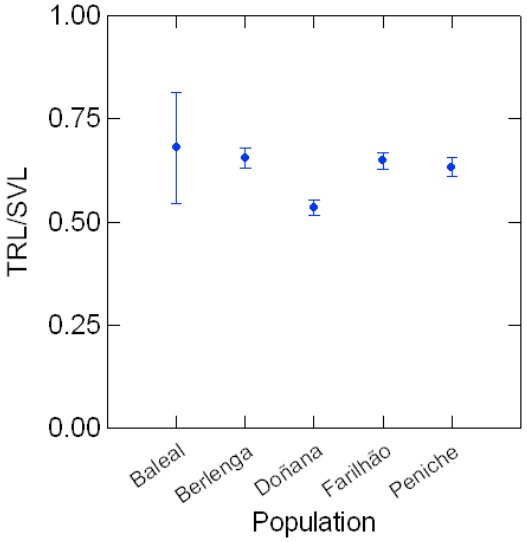

Figure 24. Plot of mean female relative Trunk length (TRL/SVL) by population. Bars represent the 95% confidence interval of the mean.

Table 30. Tukey HSD matrix of pairwise comparison probabilities for female relative front foot length (FFL/SVL). Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	1.000				
Berlenga	0.945	1.000			
Doñana	1.000	0.604	1.000		
Farilhão	0.839	0.015*	0.629	1.000	
Peniche	1.000	0.747	0.997	0.270	1.000

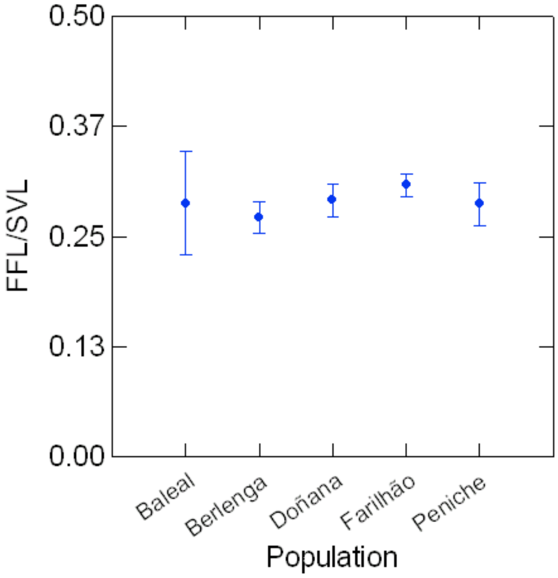

Figure 25. Plot of mean female relative front foot length (FFL/SVL) by population. Bars represent the 95% confidence interval of the mean.

In males there was no significant difference among populations in the relative Head Length (HL/SVL), relative Front Foot Length (FFL/SVL) (Table 31) or relative Pilaeus Length (PL/SVL) (Kruskal-Wallis Test Statistic =2.916, p= 0.572, df=4). The population of Farilhão had a significantly higher relative head width than the population of Peniche and Baleal (Table 32, Figure 26).

Table 31. Analysis of variance for 6 standardized morphometric variables among males. Significant results are marked with an *

Variable	F-ratio	p
HL/SVL	0.898	0.472
HW/SVL	4.717	0.002*
TRL/SVL	10.126	<0.001*
FFL/SVL	1.684	0.167
HFL/SVL	5.088	0.001*

Note: Degrees of freedom for all tests are 4, 54.

Table 32. Tukey HSD matrix of pairwise comparison probabilities for the variable relative head width (HW/SVL). Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	1.000				
Berlenga	0.425	1.000			
Doñana	0.225	0.982	1.000		
Farilhão	0.046*	0.631	0.951	1.000	
Peniche	0.983	0.389	0.149	0.004*	1.000

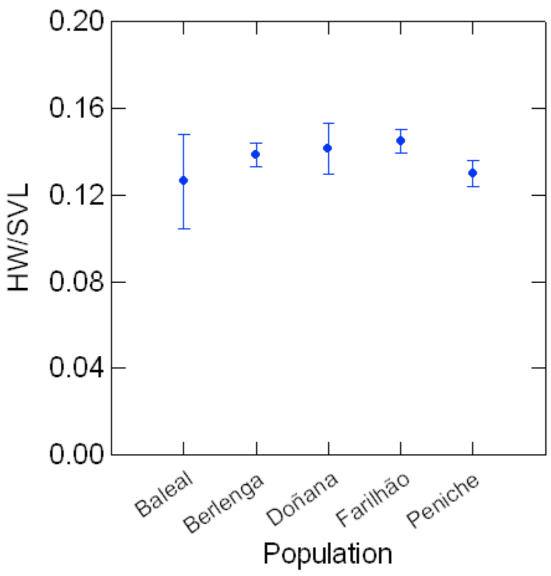

Figure 26. Plot of mean male relative head width (HW/SVL) by population. Bars represent the 95% confidence interval of the mean.

Significant differences were also found among male populations in relative trunk length. The population of Doñana had a shorter relative trunk length when compared to the other populations. Moreover, the population of Farilhão had a shorter relative trunk length than the population of Baleal (Table 33, Figure 27). The relative length of the hind foot was significantly higher in the population from Doñana when compared to the populations of Peniche and Baleal (Table 34, Figure 28).

Table 33. Tukey HSD matrix of pairwise comparison probabilities for the variable relative trunk length (TRL/SVL). Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	1.000				
Berlenga	0.181	1.000			
Doñana	0.000*	0.000*	1.000		
Farilhão	0.045*	0.969	0.000*	1.000	
Peniche	0.190	1.000	0.000*	0.860	1.000

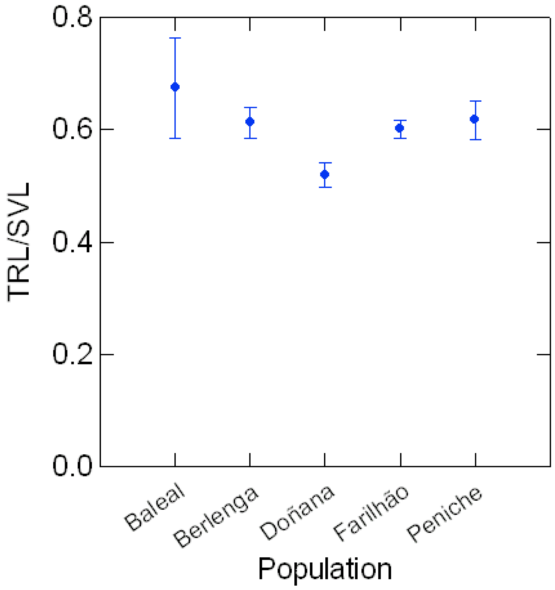

Figure 27. Plot of mean male relative Trunk length (TRL/SVL) by population. Bars represent the 95% confidence interval of the mean.

Table 34. Tukey HSD matrix of pairwise comparison probabilities for the variable relative hind foot length (HFL/SVL). Significant differences are marked with an *.

	Baleal	Berlenga	Doñana	Farilhão	Peniche
Baleal	1.000				
Berlenga	0.060	1.000			
Doñana	0.001*	0.206	1.000		
Farilhão	0.034*	1.000	0.113	1.000	
Peniche	0.108	0.978	0.036*	0.953	1.000

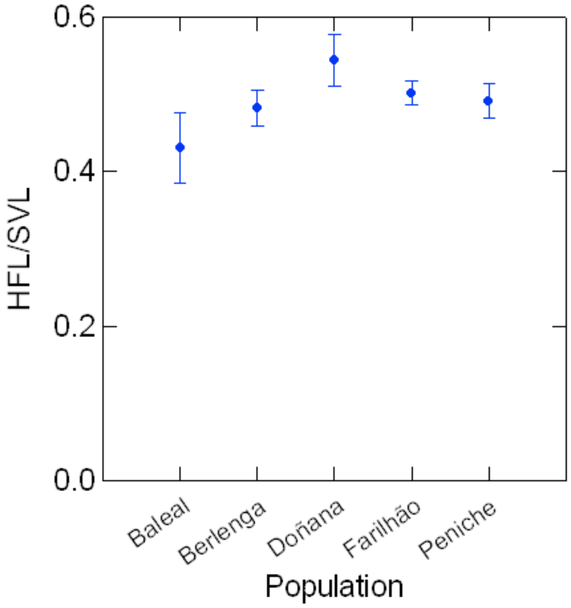

Figure 28. Plot of mean male relative Hind Foot length (HFL/SVL) by population. Bars represent the 95% confidence interval of the mean.

C. Lactate assay data

A total of 76 blood lactate concentration measurements were obtained. There was a significant effect of location on the blood lactate concentration (n= 76, df=3, F-ratio= 3.145, p= 0.031). The population of Peniche had significantly lower blood lactate concentration than the population of Berlenga (Tukey HSD, MS = 9.74, df= 68, p=0.031) and Farilhão (Tukey HSD, MS = 9.74, df= 68, p=0.049) (Figure 29) There was no significant effect of gender nor interaction between gender and location on the blood lactate concentration (n= 76, df=1, F-ratio= 1.993, p=0.163 and n=76, df=3, F-ratio= 1.408, p=0.248).

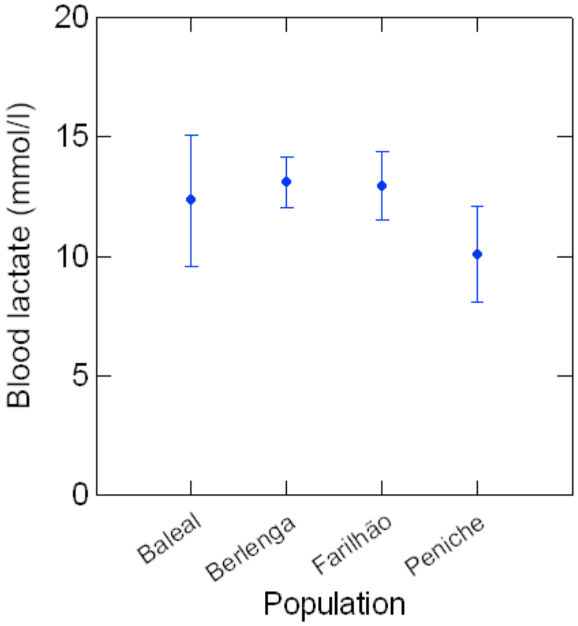

Figure 29. Blood lactate concentration (mmol/l) means by population. The bars represent the 95% confidence interval.

V. **Discussion**

A. **Genetic diversity of island and mainland populations**

The number of founder individuals, genetic drift and population bottlenecks can severely impact allele frequencies in small sized and isolated populations such as island populations (Frankham, 1997; Madsen et. al, 2000; Knopp et. al, 2007). Island populations typically have less genetic variation than mainland populations, but show higher population differentiation (Johnson et. al, 2000). In this study, one of the initial hypotheses was that the island populations would have lower genetic diversity values than the mainland populations sampled. The haplotype (gene) diversity determined using the intron 7 of the β-fibrinogen gene supported this hypothesis as higher levels of diversity were seen in the mainland populations as compared to the populations of Berlenga and Farilhão. This result was expected as previous work with isozymes had detected these trends in some of our study locations (Almeida, 1993). However, the heterozygosity values obtained in this study for both island and mainland populations were high. When comparing these values with another Iberian lizard analyzed (using the 7th intron of the β-fibrinogen gene), Schreiber's lizard (*Lacerta schreberi*), over its entire range of distribution, the maximum heterozygosity obtained was about 0.766 (Godinho et. al, 2006). This value is still lower than the maximum value obtained for our study. Furthermore, the overall heterozygosity value for *P. carbonelli* calculated with several populations in the Iberian Peninsula with mtDNA was determined to be 0.823 (Pinho et. al, 2007). This shows that despite the small geographic area of this study, which only included four populations, high haplotype diversity is found within this region.

When looking at the nucleotide diversity values, no segregation between islands and mainland is observed; the populations with highest nucleotide diversity are Farilhão and Peniche, while Berlenga and Baleal have the lowest values. The π values obtained in this study are within the expected range for eukaryotic organisms, which is from 0.02 to 0.019 (Nei, 1987). Comparing the obtained values in this study with other values reported for *P. carbonelli*, we see that the overall nucleotide diversity for *P. carbonelli,* using this same DNA region, was 0.01267, which included samples from a much wider sampling area than the one covered in this study. Such information provides a particularly interesting insight about the population of Peniche because here it is more likely that two individuals selected at random will have different nucleotide sequences, than when randomly selecting two individuals from the overall range of *P. carbonelli* in the Iberian Peninsula. Also, the population of Berlenga presents the lowest level of nucleotide diversity(which was expected due to the insular nature of this population), but Farilhão has a genetic diversity one order of magnitude higher than Berlenga. The discrepancies found in genetic diversity indices might provide us some insight about what happened to the populations is the past. Specifically, high nucleotide and haplotype diversities are indicative of a stable population (Avise, 2000), and this seems to be the case for the population in Peniche. However, low haplotype diversity and high nucleotide diversity, which appears to be the case for Farilhão (according to Grant and Bowen, 1998, $h<0.5$ is small), could be indicative of either the occurrence of a brief bottleneck in the population history, which would eliminate some of the haplotypes but would not necessarily affect the nucleotide diversity. As for the population of Berlenga, the scenario is the opposite of

Farilhão as there is higher haplotype diversity and lower nucleotide diversity. This situation could be the outcome of fast population growth initiated by the colonization of the island by a small founding group or the occurrence of a bottleneck followed by a quick population growth associated with accumulation of mutations (Grant and Bowen, 1998; Avise, 2000).

B. **Island Genetic Isolation and Structure**

Another aspect of the geographical region I analyzed was the isolation of the island populations. Several studies have determined that lizard populations on islands have limited dispersal abilities and genetic drift is responsible for the divergence found in neutral markers in these populations (Calsbeek and Smith, 2003; Jordand and Snell, 2008). The two differentiation indices calculated in this study provided different scenarios for the level of isolation present in each island. In both analyses, the consistent result was that the populations of Peniche and Berlenga were the most distinct. The high divergence values (Gst= 0.08969 and γst=0.14767) were accompanied by low estimates for number of migrants (Nm=2.54 and Nm=1.44, respectively).

Even though in this study the comparison between Berlenga and Peniche had the highest Gst (0.08969) and γst (0.14767), when looking at other intraspecific studies, these values are in fact at the low end of the range and are estimates associated with high levels of gene flow. In general, genetic differentiation values between 0 and 0.05 indicate little genetic differentiation, and between 0.05 and 0.15, moderate genetic differentiation (Hartl and Clark, 2007). In comparison, the numbers obtained for *P. carbonelli* are low (even though there are some values in the moderate range) and they indicate high gene flow. Overall, the values obtained for genetic differentiation suggest that there is very high gene flow between all populations.

The fact that Peniche and Berlenga appear to be the most distinct from all comparisons is a curious result because between the two islands only Berlenga is inhabited by humans, and it is the only island that experiences high levels of nautical traffic, particularly during the spring and summer months. This shuttling of boats between Peniche and Berlenga would seem a possible mechanism to maintain gene flow between the two locations as the lizards could accidentally enter one of the boats and be carried to the other location. However, another scenario is possible: frequent migration of lizards from the mainland to island populations and vice-versa. This has previously been demonstrated in other lizards species by Calsbeek and Smith (2003). In this study, microsatellites were used to show that *Anolis* lizards were able to migrate from one island to the other following the direction of the oceanic currents. The specifics of the migration are not known, but some lizards of the species *Anolis* species are able to survive 24 hours while floating in salt water and these lizards have also been seen entering in the ocean and swimming away from an island on their own (Calsbeek and Smith, 2003). This suggests that natural migration from one island to the other is a possibility.

To analyze this possibility in the context of my data, I measured the minimum distance the *Anolis* lizards would have to migrate, choosing one of the routes determined by Calsbeek and Smith, to get from one island to another. The minimum distance that a lizard would have to migrate in this case would be approximately 41 km, in a straight line, from Cat island to Eleuthera. The maximum distance in our study region is the distance between Baleal and Farilhão, which is approximately 21 km. The closest populations separated by the sea are the populations of Berlenga and Farilhão which are 7 km apart. The surface currents in the Atlantic ocean in this region are quite complex. Data are scarce, and Bischof et. al (2003) mentioned the lack of information regarding the spatial definition of the Portuguese Current, which makes the research on this topic even more difficult. Nonetheless, three sources were found with pertinent references to this matter. One of the sources is a document by the United States Government with sailing directions from the European West Coast (National Geospacial-Intelligence Agency, 2005). In this document, both the Berlenga and Farilhão islands are mentioned but only information regarding the ocean current in the channel between Farilhão and Berlenga is mentioned; "The channel between Os Farilhoes and Las Estelas is deep and clear, but a strong current sets from the former to the latter and great caution is necessary when navigating through it (National Geospacial-Intelligence Agency, 2005)."

Furthermore, Coelho et. al (2002) developed a model for ocean circulation on the Iberian coast and the results from their model for the year of 1994 were in agreement with other data available. They found that on the Iberian coast there is a seasonal cycle of current flow. Specifically, during the summer, flow near the shore is towards the south, but at the latitude and longitude of the channel between Berlenga and Peniche (approximately 39º 23'N and 9º 27'W) the currents flow south by the coast, but also towards the equator. In the winter, the authors found that the currents in this area were strong but much more complex and were not plotted. Finally, Bischof et. al (2003) contains plots of NOAA AOML Drifting Buoy Data Assembly (DAC) Center's archived near-surface buoys, from 1978 to June 2003, with the direction the buoy was taking. From this plot (the season is not mentioned), one can see that near the coast of Peniche, the buoys tend to float towards North, but a few kilometers away from the coast they tend to flow south.

From the data described above, one cannot accurately predict flow of currents in the study area. Nonetheless, migration along the current is possible in almost any direction (Figure 30).

Another possibility that might explain such low levels of differentiation is that not enough time has passed for genetic differentiation to occur and consequently, the populations are still very similar to each other and to the founders of the 4 populations under study. The second explanation would seem more plausible in the case of both Berlenga and Farilhão due to the fact that migration from the islands to the mainland or vice-versa would be less likely. However, in light of the information above regarding other lizards migrating through the ocean at distances at least double the distance between these populations, this hypothesis cannot be rejected.

As for the values of divergence between mainland locations, high values were also obtained, but as Peniche and Baleal are geographically close (approximately 6 km), the possibility of *P. carbonelli* migrating from Baleal to Peniche (and the opposite) cannot be disregarded.

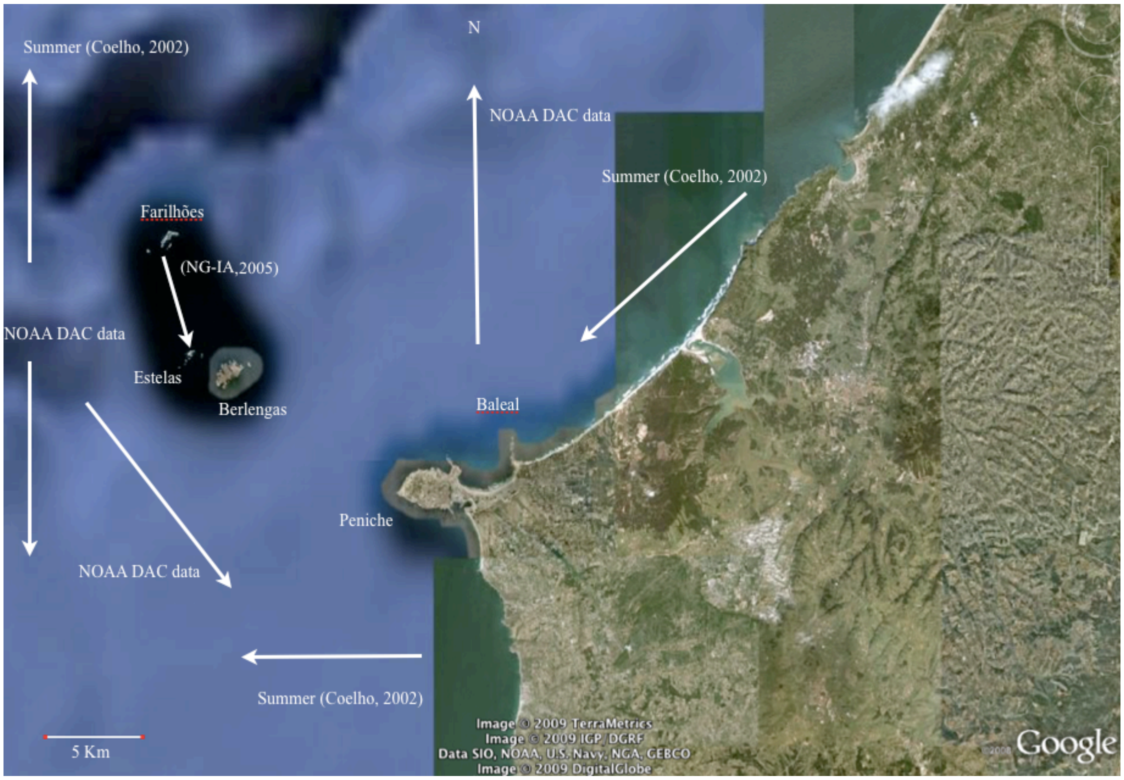

Figure 30. Map with the main ocean current directions in the study region. Arrows do not have a fixed latitude or longitude (except for the arrow between Farilhão and Estelas) as the plot is an approximate representation of the available data and not enough information is available to accurately place the ocean current directions.

According to Johnson et. al (2000), one of the genetic consequence of island biogeography is that allelic divergence increases with island size. Therefore, in the study region, one would expect the highest levels of divergence to be between mainland locations and the largest island, which is the island of Berlenga. Furthermore, genetic divergence between Farilhão and the other mainland populations is lower than the divergence with Berlenga. This suggests that either the founding members of Farilhão did not come from Berlenga but instead were from these mainland regions or that high gene flow exists between Farilhão and the mainland. Hence, despite the low differentiation, the relationship between molecular differentiation in the four regions is what would be expected according to island biogeography.

The AMOVA further supports the results discussed above. The majority of genetic variation is found within populations (88%) , while a smaller fraction is found among groups (7.22%) and an even smaller percentage among populations within groups (4.73%). Furthermore, the population structure statistics revealed low, statistically significant values, for the genetic structure within populations and among populations within groups. These results and the results discussed previously suggest that these populations are genetically very similar, with little or no divergence between islands and mainland locations.

C. Haplotype history and distribution

In this study, one of the objectives was to identify which haplotypes evolved exclusively in island populations and of these, which were most likely ancestral haplotypes and what factors shaped the current haplotype distribution. To answer these questions, a three step approach was taken. First, the haplotypes found in the study region were compared to haplotypes previously described for the Iberian Peninsula (using other locations than our study region). Second, a haplotype network was built to reveal the relationship between the haplotypes found. Finally, a nested clade analysis (Templeton, 1998) was performed to determine whether there was a correlation between the geographical distribution of the populations and the haplotypes found, and if so, what events led to the current distributions.

The haplotype network built revealed Hap_5 as the most likely root of the network (outgroup probability=0.328), and thus, the most likely ancestral haplotype. According to Castelloe and Templeton (1994), an interior haplotype has a high likelihood of being the root of the network when it has a high multiplicity (number of individuals that have that haplotype) or when it is a neighbor of a haplotype with high multiplicity. In this case, Hap_5 has a multiplicity of 3 but it is a neighbor of Hap_4, which has a multiplicity of 88. Haplotype Hap_4 also has a high outgroup probability (outgroup probability=0.308) and fulfills other criteria to be the ancestral form: it is the most common haplotype (according to coalescence theory, the rank of haplotype frequency corresponds to the rank of haplotype age), it is present in all populations sampled and it forms a start phylogeny because it is directly connected to 5 other haplotypes (while Hap_5 is only connected to 4) (Castelloe and Templeton, 1994; Avise, 2000). Hap_5 only occurs in the current island populations (Berlenga and Farilhão) and has lower connectedness (it is only connected to 4 haplotypes). This result (Hap_5 being the ancestral) is the outcome of a “neighbor effect” (Castelloe and Templeton, 1994) i.e., when an interior haplotype has the highest multiplicity from the entire sample, its probability of being the root can decrease and shift to one of the haplotypes to which it is connected to. This is most likely what happened in this case. Furthermore, since only haplotype frequency, not haplotype distributions used to calculate the network, it is more likely that Hap_4 is the ancestral haplotype due to its high multiplicity, the fact that it is present in all populations sampled, and because it has the highest number of connections to other haplotypes (older haplotypes would have more time to mutate giving rise to new haplotypes). Furthermore, when this haplotype network is built without taking into account the frequency of each haplotype (the multiplicity of Hap_4 cannot influence the outgroup probability of Hap_5), the same network is obtained but Hap_4 is identified as the root of the network in this case.

Hap_5 was found in the mainland (other places than the study region), while Hap_4 was not. Three possibilities explain the apparent exclusivity of Hap_4 (and of the other haplotypes) to the study region. It is possible that the study which deposited the sequences in GenBank (Pinho et. al, 2006) did not sample enough individuals to obtain the range of haplotypes that were obtained in this study, and consequently, some of the haplotypes actually exist on the remaining of the peninsula but were not sampled. The study by Pinho et. al (2006) did not sample the mainland close to our region so it is possible that they simply failed to obtain Hap_4 in their samples. Another possibility is that all 20 haplotypes exclusive of our region do not exist anywhere else in the peninsula. The third option would be a mix between the first two, i.e., some haplotypes were not sampled and some haplotypes are indeed exclusive of this area.

The nested clade analysis (NCA) (Templeton, 1998) allows the quantification of associations between geography of populations and phylogeny of haplotypes and provides information regarding the evolutionary processes most likely responsible for the observed association. The nested clade analysis fails to reject the null hypothesis of no association between phylogeny and geography when there is high gene flow, recent population differentiation, or when sample sizes are small or geographic sampling is inadequate (Avise, 2000). Our data set was characterized by low genetic differentiation (caused either by high gene flow or recent divergence of populations) and our sample size in terms of geographic regions was small (4 locations). Nonetheless, some of the hierarchically lower clades showed significant association between geography and phylogeny.

The distribution of haplotypes H_2 and H_1 was likely derived by restricted gene flow and isolation by distance. The star-like pattern found in clade 2-1 with haplotype H_4 in the center is characteristic of a population that has rapidly grown in numbers from a small founder group, while expanding its range. This is consistent with the historic event inferred for clade 2-1 (contiguous range expansion) and with the hypothesis of recent differentiation of our populations.

The interpretation of the results for clade 4-1 and 4-2 are complicated and most likely inappropriate due to the low number of regions sampled in this study. Both clades have haplotypes from the four populations so one cannot overlay the clades with geographical regions and make inferences regarding the restriction of gene flow between clades because the entire network does not have significant association between geography and phylogeny. Nonetheless, the haplotypes that are in each clade, which are exclusive of a population, are so due to restricted gene flow and isolation by distance.

It has been mentioned previously that the NCA would not show any association between geography and phylogeny when either gene flow was high or when population differentiation was recent. As the main historical event inferred from the observed distribution of haplotypes is restricted gene flow with isolation by distance, in clades with significant association between phylogeny and geography, these results further support the hypothesis of recent genetic differentiation of these populations.

D. Combining mtDNA and nDNA results

The results obtained for both mtDNA regions (control region and 12S RNA) appear contradictory to the results found with the intron 7 of the β-fibrinogen gene. All individuals sampled for the control region and for the 12S RNA region had the same haplotype, while a total of 22 haplotypes were found in the bases upon nuclear intron sequencing. This situation appears to be a consequence of two factors. One is an intrinsically low mtDNA diversity in *Podarcis carbonelli* and the other is the high degree of polyphyly seen in this nuclear DNA region in all Iberian *Podarcis*. Pinho et. al (2007) has described *P. carbonelli* as a species with intermediate mtDNA genetic diversity, with a shallow geographic structure over the Iberian Peninsula. As my study region was so small, it is very likely that the area only has a few mtDNA lineages, therefore explaining why all individuals sampled were part of the same lineage. The nuclear intron has much higher diversity levels in this geographic region, and the different haplotypes do not from a clear geographical structure as it has been previously discussed. Pinho et. al (2008) has found a surprising lack of monophyletic species when it comes to defining Iberian Podarcis using this nuclear region because sequences found in one species are more similar to sequences found in other species, as opposed to the sequences found in a single species being the most similar. This is most likely what is happening in our populations at a lower taxonomical scale. The sequences found in the different populations are not more similar within populations, but between populations, resulting in a lack of genetic structure. Therefore, two scenarios could originate the situation described above: high levels of gene flow or incomplete lineage sorting.

High levels of gene flow in a species with low mtDNA haplotype diversity would keep the same mtDNA haplotype in all populations, and maintain the existing nuclear haplotype diversity observed. This explanation is less likely due to the information obtained from the nested clade analysis in conjunction with the population differentiation indices. Even though migration among the populations could be a reality, the nested clade analysis points toward restricted gene flow and isolation by distance, which explains the occurrence of several nuclear haplotypes specific to a single population.

The other alternative is incomplete lineage sorting. It is possible that other mtDNA lineages were present in these regions at a time, but through genetic drift one haplotype became fixed in all four populations. However, nuclear DNA genes take longer to become fixed in a population. Specifically, two nuclear alleles will take on average four times longer to become fixed in a population than two mtDNA haplotypes (Moore, 1995). This alternative explains the complete lack of mtDNA diversity (based on the two markers sampled) and the observed nuclear DNA diversity, while corroborating the hypothesis of recent population differentiation.

E. Morphological differences

Insular populations are known to show conspicuous morphological differences from their mainland conspecifics. It has been suggested that this morphological differentiation can arise due to mutation and genetic drift (random microevolutionary processes) (Wright, 1931). However, Clegg et. al (2002), by testing the hypothesis of random microevolutionary events being responsible for phenotypic differentiation in *Zosterops lateralis*, have found that it is likely that directional selection is causing the phenotypic differentiation instead. The morphological feature that most commonly changes in insular populations of a species is adult body size (Keogh et. al, 2005). There are several possible explanations as to why an insular population might show gigantic or dwarf forms, including the decrease in predatory pressure, decrease in competition, genetic drift, as well as size of available prey items (Keogh et.al, 2005).

The morphological differentiation of *P. carbonelli* from Berlenga and Peniche when compared to other mainland regions has been previously documented (Vicente, 1989; Sá-Sousa et. al, 2000). However, in the context of this study, it becomes pertinent to approach this matter in all four populations as molecular data suggests that these populations are not so distinct.

The analysis of morphological characteristics of the four populations and the distant population of Doñana revealed that some of these populations, in terms of their size, are clearly distinct from the others. The population of Farilhão, both males and females, were statistically different from all others based on weight, length and body condition. These results were not surprising as during sampling these were the biggest individuals captured from all populations. It appears that there is a gradient in terms of size measurements (in both genders) when looking at the four populations. Lizards from Farilhão are the largest and most robust, followed by the population of Baleal and

Berlenga (not statistically significant from each other), Peniche (significantly smaller than Berlenga) and Doñana, the smallest and least robust particularly in the case of males. This is a curious result since the populations that are genetically most different are Berlenga and Peniche, yet in terms of size it seems that Farilhão is the most distinct. This result suggests that the mechanisms in place causing the genetic divergence seen in these populations are likely not the same causing the morphological differences, otherwise, as the genetic marker used is neutral, one would expect to see the same divergence pattern when analyzing both molecular and morphological data (Clegg et. al, 2002). There are three processes that might explain this difference in size seen in our populations. One of the explanations might be that the organisms in Farilhão are on average the oldest and therefore the largest. Reptiles experience continuous growth, and so as a lizard ages, its body size will also increase (Shine and Madsen, 1993). This hypothesis was not tested as direct age-determination methods in lizards are through skeletochronology. Another possibility is that all lizards have on average the same age but have adapted to their current environment and have acquired new morphologies that are the outcome of a distinct genetic configuration. The third possibility is that this is a case of phenotypic plasticity in which factors such as food availability cause the observed size differences between populations (Shine and Madsen, 1993). With the current data it is not possible to distinguish between these three hypothesis for the size differences seen in the populations.

Overall, absolute body measurements were good predictors as to which population a lizard belonged to except for the population of Baleal. This inability of discriminating the individuals from Baleal was likely due to the low sample size for this population (females n=2, males n=4). When body proportions were used instead, the classification accuracy of the canonical variates analysis decreased but it was still possible to determine the population of origin of the individuals accurately, 81% of the time in females and 68% in males. This means that there is not just a size component to the differentiation but the size of the different body parts, relative to the overall length of the lizard is diagnostic of each location as well (except for Baleal).

The results of the cluster analysis further support the hypothesis that the mechanisms responsible for genetic differentiation are not the same as the mechanisms for morphological differentiation. Although caution is necessary when interpreting these results due to the low sample size of Baleal, and due to the fact the cluster analyses were performed on the mean canonical scores of each population and not on the canonical scores of each individual, some interesting trends appeared.

With regards to absolute and relative body measurements in females, when clustering populations based on similarity the populations of Peniche and Baleal were the most similar, followed by Berlenga and Farilhão. Even with low sample sizes, this result is consistent with the hypothesis that insular populations will show more divergence from mainland populations. Therefore, it is not surprising that the two mainland populations are the most similar in terms of size and body proportions.

The male populations, however, revealed a different trend. The populations of Berlenga and Peniche were the most similar and the populations of Donãna was the most different. These results were somewhat unexpected as they are not consistent with the results from females or what would be expected from the theory of island biogeography (higher differentiation between island and mainland populations) (Johnson et. al, 2000). The male population of Doñana was the smallest of all populations, according to the ANOVA performed. The higher similarity between the male absolute body measurements of Berlenga and Peniche could be attributed to convergence due to similar selective forces. As this trend is only seen in males, it seems reasonable to speculate that the force driving the similarity of size is related to a male-specific trait, such as territoriality or other male-specific behaviors. Without more information of population densities in Peniche it is difficult to explain this similarity in size, but if lizard densities are similar in the two locations, it can be assumed that the territory defended by males will be of similar size. If the territory is of similar size in both populations, all else assumed equal, the lizards would be consuming the same amounts of food which would constrain their size in a similar fashion. However, this is a speculative explanation.

However, another explanation has to be considered: one related to the low sample size of Baleal. The low sample size might have provided an erroneous representation of the similarity of Peniche and Baleal. With an adequate sample, these two populations might be the most similar and cluster together in this analysis.

The cluster analysis with male body proportions revealed another trend not yet seen: the populations of Berlenga and Farilhão were the most similar. Looking at these results together with the one-way ANOVAs for male relative proportions, the trend is that the populations of Berlenga and Farilhão are not significantly distinct for any of the measurements. This suggest another scenario of convergence between Berlenga and

Farilhão with regards to body proportions. Other studies with Anolis lizards have shown that differentiation between populations with regard to body proportions can be related to different habitat selection (Calsbeek and Irschick, 2007). In this case, it is likely that the opposite is happening. The habitat occupied by *P. carbonelli* in Farilhão is distinct from the habitat in Berlenga. Farilhão is a very steep island, and the substrate is coarse sand, as opposed to Berlenga, which has a flat top and the substrate is finer soil, so based on topology of the island, one would expect these populations to diverge in terms of body proportions. However, in terms of vegetation, both islands have similar plant species, and the lizards usually take advantage of the vegetation to hide. The behavior of hiding underneath dense patches of *Carpobrotus sp.* was seen in both islands during the sampling season. It is possible that the body shapes from male from Berlenga and Farilhão are converging due to a similar use of habitat that favors a specific shape.

Other relative body measurements that are relevant to mention are the narrower head of females and males in Peniche when compared to Farilhão and Doñana; the shorter trunk of females and males from Doñana, and the longer hind foot of the Doñana males, when compared to Peniche and Baleal. These differences in body proportions are most likely related to differences in behavior that favor different morphologies. However, caution must be present when inferring the possible causes behind these differences as no rigorous behavioral observations were made during this study. The narrower head of females in Peniche, was also seen in males of this population so it is likely that whatever pressure is pushing this population towards a narrower head, it is not gender specific. This narrower head could be associated with the behavior of this population to hide in stone walls. The habitat in which the Peniche lizards were found were average-density vegetation patches along a castle wall with crevices. Almost all lizards seen during sampling would use these crevices to hide or to bask with the protection of the wall. Therefore a head adapted to fitting in narrow crevices would seem advantageous for both genders in this population

In the population of Doñana both genders showed a significantly smaller relative trunk length. This population was also the smallest in terms of weight, SVL and body condition. This population is considered a subspecies of *P. carbonelli* and is isolated in the sandy areas of a small region in Southern Spain, which biogeographically would resemble an island. It is possible the lizards in Doñana suffer from insular dwarfism. Other species of reptiles, instead of showing a shift in body size towards larger proportions, present a body size smaller than their mainland conspecifics (Keogh et. al, 2005). As these lizards were, in both genders, consistently smaller than both the large island forms and the mainland populations, it is likely that this is a case of dwarfism. Another interesting feature about this population was that the males had significantly larger relative HFL than Peniche and Baleal. The populations in Peniche and Baleal inhabit rocky soils or flat soils with earth while in Doñana these lizards live exclusively in the sand. A longer hind foot would be advantageous to these individuals for both cooling rapidly, as longer extremities allow faster dissipation of heat, and to move more easily on sand due to the larger surface occupied by the hind leg and foot.

F. Population Lactate levels

One of the assumptions associated with the insular gigantism effect is that predatory pressure on islands is usually lower than in the mainland (Keogh et. al, 2005). Lactic acid is a byproduct of the anaerobic metabolism and in lizards most of the locomotor activity associated with escaping predators is fueled by this type of metabolism (Bennett, 1994; Wagner and Gleeson, 1997). Based on this information and the possible lower predatory pressure on islands, I formulated the hypothesis that the lizards on islands would be under lower predatory pressure and would show lower levels of blood lactate concentration when compared to mainland populations.

The analysis of the blood lactate concentrations revealed a trend different from what was expected according to my hypothesis. The population of Peniche has a lower blood lactate concentration than both island populations. Several possibilities could explain these results. The first is that predatory pressure is not lower in these islands when compared to Peniche. Both the Berlenga and Farilhão Islands have a high population of seagulls (*Larus sp.*) (ICNB, 2007). It has been debated whether seagulls act as predators of lacertid lizards. On the one hand, the seagulls may deter other predatory birds from approaching the ground and also, it has been hypothesized that during the nesting season seagulls might refrain from attacking lizards because these might help reduce the ectoparasitic load on the chicks (Vervust et. al, 2007). On the other hand, other studies have reported increased predatory pressure in *Podarcis atrata* when seagulls are present (Vervust et. al, 2007).

Peniche also has seagulls but in lower numbers than Berlenga and Farilhão. They were not seen in the location where the lizards were sampled, as opposed to Berlenga and Farilhão, where seagulls were nesting next to the sampling sites, on the ground. Furthermore, the Portuguese Red Book of Vertebrates mentions seagulls as on of the threats to these lizards in the Berlengas archipelago (ICNB, 2006). These observations suggest that predatory pressure in both islands (if seagulls prey upon the lizards) is higher than in Peniche, and that would explain the lower blood lactate concentrations in this location. If there is lower predatory pressure, the lizard will not engage in physical activities powered by anaerobic metabolism as often, which would result in consistently lower blood lactate concentrations.

Another possibility that could explain the difference in these populations is related to habitat structure and home range. It was mentioned previously that the lizards in Peniche occur along a castle wall in flower beds of a municipal park. During the sampling season, these flower beds have dense vegetation, which provides both cover and basking spots for the lizards, and so does the castle wall. The space between the castle wall and the end of the flower bed is about 50 cm wide. During sampling, lizards were not seen outside this space and would not move more than 1.5 meters in either direction, which makes the area they occupied relatively small. The lizards in Berlenga and Farilhão however, where seen moving within wider areas as their habitat is not as structured as the habitat in Peniche. Lizards were seen moving from vegetation patches, to basking locations that were around 2 meters away from each other. Also, some studies have demonstrated that as body size increases, so does the home range of a lizard

(Schoener and Schoener, 1982; Perry and Garland Jr, 2002). In our study, we have demonstrated that the lizards in Berlenga and Farilhão are significantly larger than the lizards in Peniche, therefore it is possible that there is also a difference in home range. If so, this means that larger lizards would have to spend more time searching for food, females and basking sites over a larger area, and consequently spend more energy during these activities and produce more lactate.

The population of Baleal did not show significantly different lactate concentration levels than the other populations, which was likely an outcome of its low sample size. Therefore, with a higher sample, it might be the case that this population would show differences from Peniche or from the islands.

G. Conclusion

This study has the purpose of assessing the populations of *P. carbonelli* in the Berlengas Natural Preserve, as well as in the two mainland locations of Peniche and Baleal. Using molecular data we found that the populations are fixed for the mtDNA regions of 12sRNA and the control region, but present high levels of diversity at the nuclear level. The mainland populations were genetically more diverse than the island populations but there was a lack of geographic structure. Both island populations appear to have been colonized by lizards from mainland populations, as opposed to lizards migrating from the mainland to Berlenga, and from Berlenga to Farilhão. These four populations appear to have diverged recently, which results in a high number of shared nuclear haplotypes among populations and a reduced number of population-specific haplotypes, which most likely were formed due to isolation by distance and restricted gene flow between populations.

Despite the likely young age of these populations, enough time has passed for significant morphological differences to arise. The island populations are significantly larger than mainland populations, and the population of Doñana appears to be suffering from a dwarfism effect. The nature of the differences between Peniche, Baleal, Berlenga and Farilhão is unknown, but age, genetic divergence and phenotypic plasticity are likely candidates to explain these differences. Genetic drift does not seem as a likely cause for these differences as the patterns revealed by the nuclear intron do not match the morphological trend seen.

Finally, significant physiological differences were found, expressed by distinct levels of blood lactate concentrations in the population of Peniche, which could be the outcome of lower predatory pressures on this location when compared to the islands, and thus lower levels of anaerobic activity in these lizards, or due to differences in home ranges and habitat structure, which would also affect the metabolism of these lizards.

From a conservationist perspective, the island populations, despite the existing nuclear diversity, show no mtDNA diversity, which could become problematic if the remaining of the mtDNA is fixed in these populations as well. The mtDNA has the genes coding for the intervenients in the oxidative phosphorylation pathway of cellular respiration. If there is an environmental change that requires a more efficient oxidative phosphorylation pathway, these populations will have to wait for a mutation to provide a new allele as there is no mtDNA haplotype variation for selection to act upon. Therefore, if a reintroduction becomes necessary in the future of these island populations, it is suggested that the introduced individuals are selected from regions with a mtDNA lineage distinct from the one present in the islands.

Furthermore, the information provided from the blood lactate concentration data suggests that in terms of predatory pressure, the island populations might be under higher predatory pressures than previously assumed.

The current conservation status of *P. carbonelli* is Endangered, for IUCN, and Vulnerable, for the Portuguese Institute for the Conservation of Nature and Biodiversity (ICNB, 2006). The global trend is that population numbers are declining (IUCN, 2009). In the island populations, due to their isolation, the absence of mtDNA diversity and the possibility of higher predatory pressure, it is suggested that population census are performed regularly as to monitor the demographic trend in these locations closely.

The morphological uniqueness of the populations in the islands deserves further study, specifically to determine its nature. Alternative techniques for age determination in lizards, that do not cause permanent physical damage to the populations, would be ideal to determine whether the lizards on islands are older than their mainland conspecifics. To determine the possibility of phenotypic plasticity being the cause of these differences, ecological studies focusing on diet, home ranges and habitat structure would help to determine the cause behind gigantism of the island populations. Finally, if the gigantism observed is caused by genetic mutations, it might prove to be complex to pinpoint which gene or genes would be responsible for such phenotype and thus testing the previous hypotheses would allow to researchers to obtain an answer by excluding easily testable hypotheses.

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