



Unravelling the sex determination patterns in a temperature sex dependent gecko species

Valéria Marques

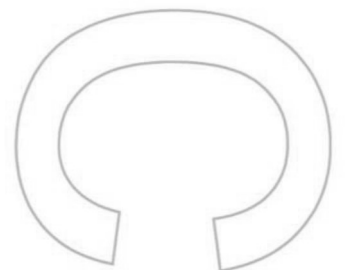
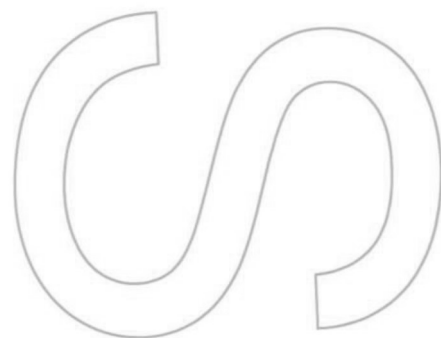
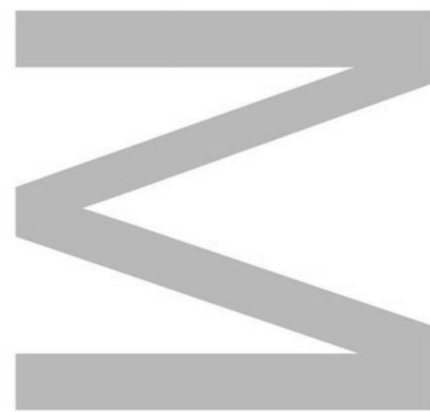
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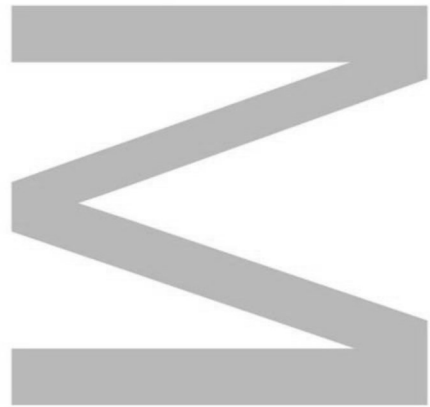




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____ / ____ / ____



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Abstract

Temperature sex determination is a mechanism in which the sex of individuals is settled by the temperature experienced during embryonic development. This mechanism may exist because it appeared in the past and has been adaptively neutral and maintained in extant lineages, or because it is adaptively advantageous. Depending on ecological and physiological traits and evolutionary potential, species with temperature sex determination may be at risk of demographic collapse due to biased sex ratios induced by fast-paced contemporary climate change. Fluctuating asymmetry, a trait connected with individual fitness, serves as a measure for embryonic developmental instability and, therefore, deviations from optimal incubation conditions. Using the Moorish gecko, *Tarentola mauritanica*, as a model, the goals of this thesis project were to confirm the existence of temperature sex determination in this species and, if true, to describe its pattern and pivotal temperature, to assess the existence of local adaptation of female nest-site choice and pivotal temperature to local environments and of different patterns of fluctuating asymmetry at the population level as indication of developmental instability or heritability. A total of 80 gecko couples from four different populations across their range in the Iberian Peninsula were collected and kept in captivity during their reproductive season. Data loggers were placed in nest sites in each population locality. Eggs were incubated at different temperatures. Fluctuating asymmetry levels of adults and hatchlings were assessed. The results confirm the existence of temperature sex determination in *Tarentola mauritanica* with a male-to-female pattern. A mostly conservative thermal ecology of reproduction in this species, with no local adaptation of female nest-site choice. There is no evidence of heritability of asymmetry and different fluctuating asymmetry patterns among populations are found, but with no connection to local incubation temperature conditions. Despite the general limited sample size, this project conveys interesting results and promising future research prospects.

Key words

Tarentola mauritanica, temperature sex determination, nest-site choice, adaptation, climate change, fluctuating asymmetry

Resumo

A determinação sexual por temperatura é um mecanismo no qual o sexo dos indivíduos é estabelecido pela temperatura experienciada durante o desenvolvimento embrionário. Este mecanismo pode existir porque apareceu no passado e tem sido adaptativamente neutro e mantido em linhagens existentes, ou porque é adaptativamente vantajoso. Dependendo de características ecológicas e fisiológicas e potencial evolutivo, espécies com determinação sexual por temperatura poderão estar em risco de colapso demográfico devido a proporções sexuais tendenciosas induzidas pelas rápidas alterações globais contemporâneas. A assimetria flutuante, uma característica relacionada com aptidão individual, serve como medida para instabilidade de desenvolvimento embrionário e, portanto, variações de condições de incubação ótimas. Utilizando a Osga Moura, *Tarentola mauritanica*, como modelo, os objetivos deste projeto de tese foram confirmar a existência de determinação sexual por temperatura e, se verdadeiro, descrever o seu padrão e temperatura pivotal, avaliar a existência de adaptação da escolha de site de oviposição pelas fêmeas e da temperatura pivotal a ambientes locais e de diferentes padrões de assimetria flutuante ao nível populacional como indicação de instabilidade no desenvolvimento ou hereditabilidade. Um total de 80 casais de geckos de quatro diferentes populações da sua extensão na Península Ibérica foram recolhidos e mantidos em cativeiro durante a época reprodutiva. Foram colocados data loggers em ninhos de cada população. Ovos obtidos foram incubados a diferentes temperaturas. Foram registados níveis de assimetria flutuante de adultos e recém-nascidos. Os resultados confirmam a existência de determinação sexual por temperatura em *Tarentola mauritanica* com um padrão macho-para-fêmea. É sugerida uma ecologia termal de reprodução maioritariamente conservativa nesta espécie, sem adaptação local da escolha de temperatura de oviposição pelas fêmeas. Não há provas de hereditabilidade de assimetria e são encontrados padrões de assimetria flutuante diferentes entre populações, mas sem ligação com condições de temperatura de incubação locais. Apesar da geral pequena quantidade de dados, este projeto transmite resultados interessantes e perspectivas de pesquisa futura promissoras.

Palavras chave

Tarentola mauritanica, Determinação sexual por temperatura, escolha de site de ninho, adaptação, alterações climáticas, assimetria flutuante

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Population: PT Oviposition temperature = $-2824.192 + 0.0654 * x$

Population: EV Oviposition temperature = $-5091.5404 + 0.1173 * x$

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Population: PT - FA = $36 * 1 * \text{Normal}(\text{Location} = -0.0278; \text{Scale} = 0.4463)$;

Population: TV - FA = $41 * 1 * \text{Normal}(\text{Location} = -0.0244; \text{Scale} = 0.8212)$.

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List of abbreviations

SDM - Sex Determination Mechanism

GST - Genotypic Sex Determination

ESD - Environmental Sex Determination

TSD - Temperature-dependent Sex Determination

TSP - Thermosensitive Period

MF - Male-Female pattern of TSD

FM - Female-Male pattern of TSD

FMF - Female-Male-Female pattern of TSD

TRT - Transitional Range of Temperature

CTE - Constant Temperature Equivalent

FA - Fluctuating Asymmetry

DS - Developmental Stability

AY - Ayamonte population of *Tarentola mauritanica*

EV - Évora population of *Tarentola mauritanica*

PT - Portimão population of *Tarentola mauritanica*

TV - Torres Vedras population of *Tarentola mauritanica*

LFF - Lamellae of the Fifth Finger

SS - Sum of Squares

MS - Mean Squares

GLM - Generalized Linear Models

AIC - Akaike Information Criterion

Introduction

1. Temperature sex determination

Sex determination is a crucial biological process which may be implemented through highly diverse mechanisms. Some genes trigger sex determination when active - they can be either allocated in special chromosomes with asymmetric pairing and partial lack of recombination (e.g. sex chromosomes) or elsewhere, but they can also be activated permanently or temporally by environmental features. Genotypic sex determination (GSD) occurs when sex is established at conception by genetic factors. Contrarily, under environmental sex determination (ESD), sex is established in response to a non-genetic factor (e.g. nutrient availability) experienced within a discrete period after conception (Bull, 1983). Across the phylogeny, most amniote vertebrates present GSD, apart from a small but highly diverse and widely distributed set of taxa in this clade. In these animals, ESD occurs as temperature-dependent sex determination (TSD): the sex of individuals is determined by the temperature experienced during embryonic development (Bull, 1980; Janzen & Paukstis, 1991; Valenzuela & Lance, 2004). This mechanism of environmental sex determination was first described in 1966, in Dakar, Senegal by Madeline Charnier, for the rainbow agama lizard, *Agama agama*, after the observation of biased sex-ratios related to different incubation temperatures (Charnier, 1966). The most well studied TSD mechanisms occur in many teleost fish, as well as in three main taxonomic groups of reptiles: turtles, crocodiles, and squamates; among the latter, it is not found in snakes. Crocodylians belonging to phylogenetically distant groups (true crocodiles, alligators and gharials) and sphenodontians (a single extant species) have exclusively TSD, whereas squamates and turtles exhibit both GSD and TSD, the latter being much more common in turtles (Janzen & Phillips, 2006; Sabath *et al.*, 2016).

However, it is important to distinguish TSD from differential gender mortality of embryos or juvenile stages, known to occur in lizards (Wapstra *et al.* 2009), birds (Göth & Booth, 2005) and mammals (e.g. wild boar, Servanty *et al.*, 2007), where contrarily to genuine ESD, here there is a decrease in total fertility.

Contrarily to a strict dichotomy between TSD and GSD, some authors (Sarre *et al.* 2004) defend the idea that these two sex determining mechanisms should represent two ends of a continuum. This view is supported by the observation that in the Australian central bearded dragons, *Pogona vitticeps*, high incubation temperature

overrides GSD, creating a female bias, with differential mortality being ruled out (Quinn *et al.*, 2007).

The time window during development at which temperature affects the sex determination is called the Thermosensitive Period (TSP). TSP generally occurs early on in the incubation period in squamates, when compared to other TSD species, with the offspring sex being determined either while the eggs are still inside the female's body or soon after oviposition, providing substantial maternal control over incubation temperatures at this critical period (Shine *et al.*, 2007). The proximate mechanisms of this sex determination are poorly understood, but experiments carried out with different model species, including turtles, crocodylians and lizards, have demonstrated the key role played by aromatase (the enzyme complex that helps convert androgens to estrogens) in gonadal sex differentiation during TSP and that the regulation of aromatase gene transcription is multifactorial and could be, directly or indirectly, influenced by temperature during the TSP (Pieau *et al.*, 1999).

There are three recognized patterns of TSD found in vertebrates, defined by the sex-ratios produced as a function of constant incubation temperatures. Two of them have a single transition zone. In one, lower temperatures result in males and higher temperatures in females (MF) and in the other, lower temperatures result in females and higher temperatures in males (FM). The third pattern involves two transition zones, with intermediate temperatures resulting in males and extreme temperatures in females (FMF) (Figure 1; Pezaro *et al.*, 2016). Another important component of TSD is the transitional range of temperatures (TRT), the range of temperatures, defined in terms of constant-temperature incubation, that produce mixed offspring sex ratios, in variable proportions; however, this component is not to be confused with the pivotal temperature, the threshold temperature at which the shift in sex ratio occurs (Bull, 1980, Hulin *et al.*, 2009).

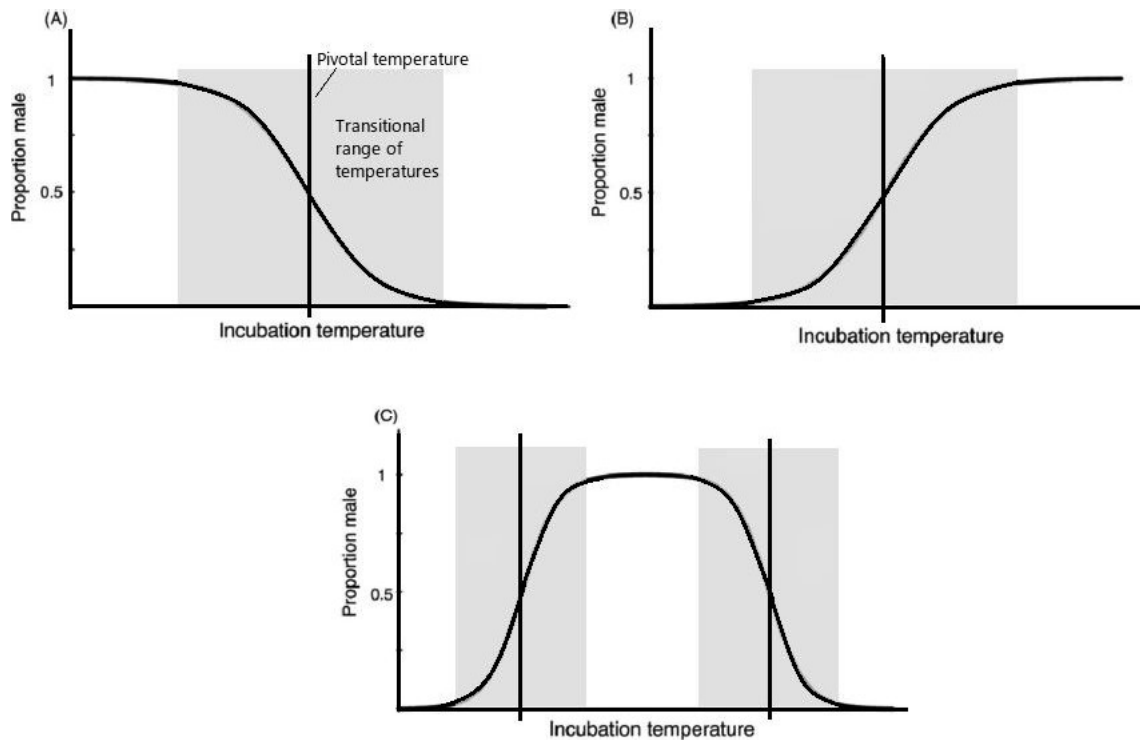


Figure 1 - Schematic illustrations of the three general patterns of TSD found in reptiles. A - males developing at cool incubation temperatures and females at warm temperatures (MF pattern); B - females developing at cool incubation temperatures and males at warm temperatures (FM pattern); C - female development at both cool and warm temperatures with males at intermediate temperatures (FMF pattern). The vertical black line represents the pivotal temperature, while the grey rectangles represent the transitional range of temperatures (adapted from Pezaro *et al.*, 2016).

Although these patterns are informative for understanding traits like the pivotal temperature, results from constant-temperature incubation experiments in the lab may not fully represent what occurs under natural conditions, where incubation temperatures fluctuate both daily and seasonally (Georges *et al.*, 2004). Methods to translate the results from constant temperature lab experiments are needed, so that accurate predictions are suitable. The Constant Temperature Equivalent (CTE) condenses thermal variation into a single number that can be used to predict the sex-ratios of species with TSD, conceiving accurate predictions about animals in the wild (Georges *et al.*, 1994; Orchard, 1976). Furthermore, it is hard to distinguish between FMF and the other two TSD patterns in experimental settings, because it is necessary to be certain about the entire temperature range for egg incubation (e.g. González *et al.*, 2019).

1.1. Adaptive advantages of TSD

Examining how populations adjust vital processes and life history traits to local environments can give perception into spatial and temporal patterns of local adaptation. Sex-ratio is an important demographic parameter, and in TSD species it can be directly impacted by climatic conditions (Janzen, 1994; Tucker *et al.*, 2008). For this reason, a powerful approach for studying adaptation is to use as model species reptiles with TSD, comparing wild populations that experience a wide range of local conditions, because population sex-ratio is potentially influenced by local climate (Doody, 2009).

The adaptive significance of TSD is still under debate. Group adaptation (adaptive control of sex-ratio, promoting group fitness) and inbreeding avoidance (single-sex clutches avoiding inbreeding) are two possible explanations. These are, in theory, plausible, but there is little to no practical evidence to support them (Janzen & Phillips, 2006). A non-adaptive explanation is phylogenetic inertia: the idea that TSD is simply maintained in extant lineages because it appeared in the past and now is adaptively neutral (or at least compensated by other traits), meaning it could have been maintained simply because the organisms who had this trait were able to survive. A phylogenetic comparative analysis implies a single origin for TSD in most amniotes around 300 million years ago, with the re-evolution of TSD in squamates after they had independently developed GSD (Figure 2; Janzen & Krenz, 2004).

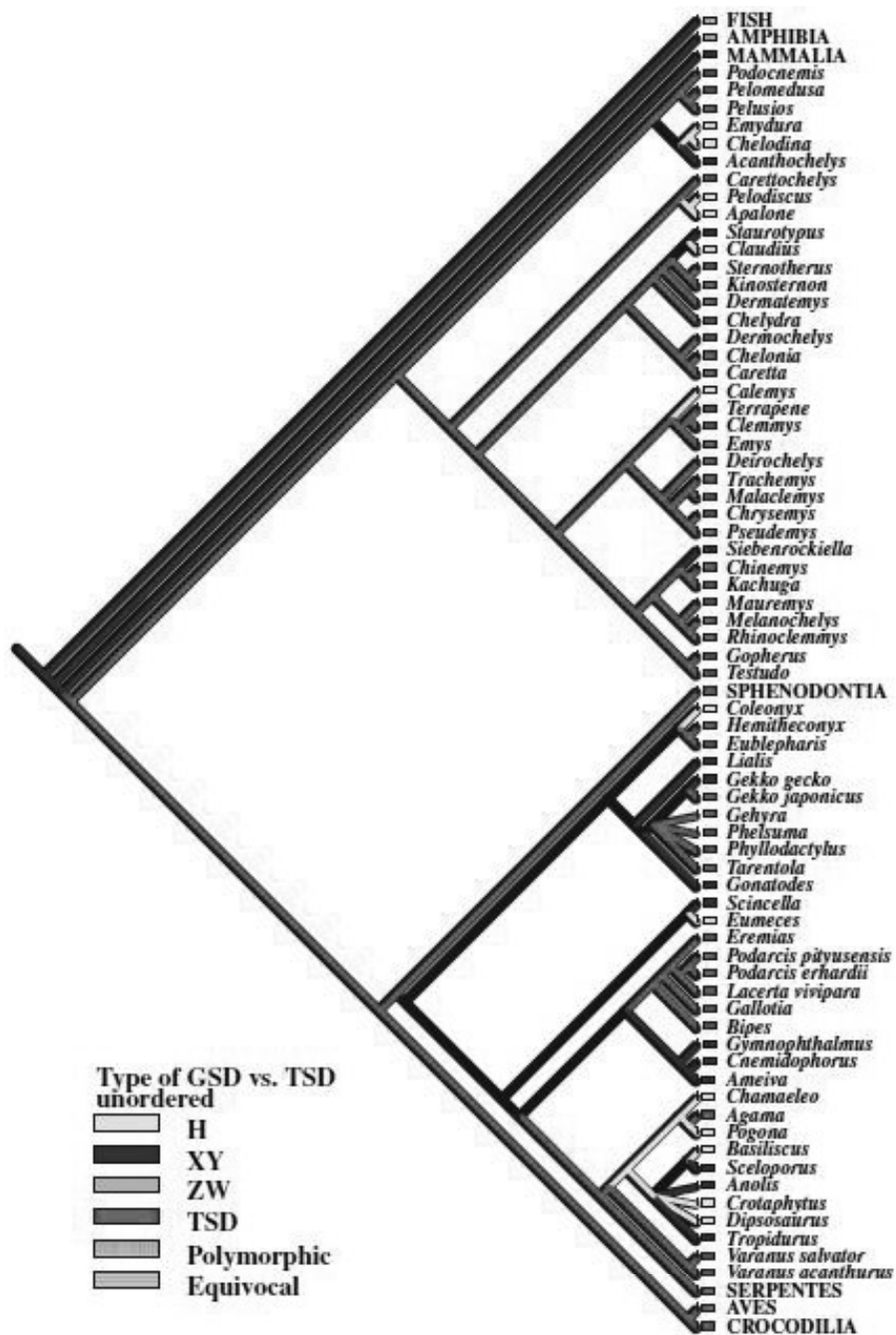


Figure 2 - Parsimony analysis of SDM evolution on a tetrapod vertebrate phylogeny (Janzen & Krenz, 2004).

Taxa with TSD are represented in red.

The most studied adaptive explanation is differential fitness, which suggests that TSD provides sex-specific fitness benefits. Charnov & Bull (1977) proposed that this type of sex determination is favoured by natural selection if an individual's fitness is influenced by environmental conditions and the individual has little control over which

environment it will experience - if an organism finds itself in an environment where male fitness is higher than female fitness, selection will favour it becoming a male. Contrary to GSD systems, this gives the individual some degree of control over offspring fitness, which may be beneficial when it comes to adaptive selection. This key aspect of the model requires the ratio of male to female fitness to vary with temperature for TSD to evolve adaptively (Figure 3; Janzen & Phillips, 2006).

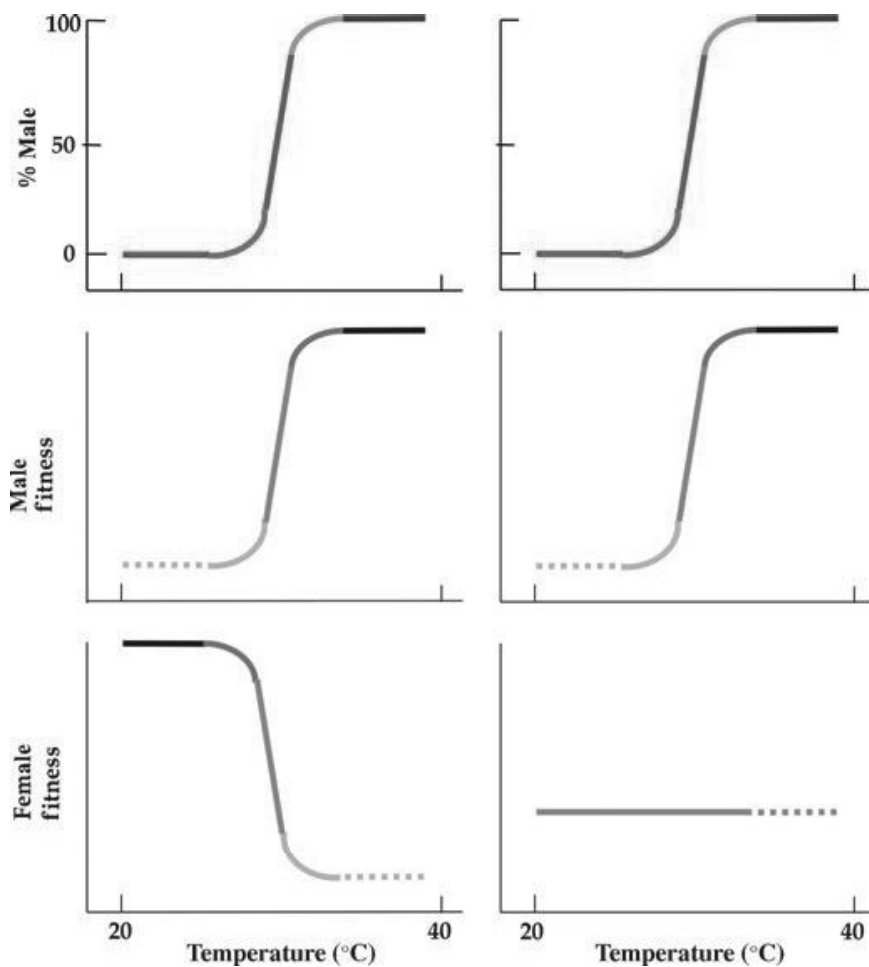


Figure 3 - Visualizations of the differential fitness condition of the Charnov-Bull model (Charnov & Bull, 1977). Based on the model, in a FM pattern of TSD (top plots), male fitness is expected to be greater than female fitness under conditions that produce male offspring (center plots), while the contrary (bottom left plot) or independence from temperature (bottom right plot) is expected from female fitness. Dashed lines indicate that individuals of the respective sex are not produced (Janzen & Phillips, 2006).

The first unequivocal empirical support for the Charnov-Bull model was provided by Warner & Shine (2008) who showed, using as model species the jacky dragon, *Amphibolurus muricatus*, a short-lived agamid with TSD, that incubation

temperatures affect reproductive success of males differently than that of females. By incubating eggs at different sex-yielding temperatures and hormonally manipulating embryo sex, after quantifying lifetime reproductive success of the lizards, it was clear that the fitness of each sex was maximized by the incubation temperature that produces that sex.

In addition, Pen *et al.* (2010) showed that, in certain populations of spotted skink, *Niveoscincus ocellatus*, a small lizard from Tasmania, it is advantageous to produce females earlier in the reproductive season - the warmth in the early season produces female-biased clutches which, in turn, develop and reach maturity earlier and possibly reproduce the same year. However, populations at different climatic extremes of this species' distribution differ in their sex-determining mechanisms, with TSD in lowlands and GSD in highlands. While in the lowlands, temperatures are more constant and there is a longer activity season, which are favorable conditions for TSD, the highlands are colder with a higher magnitude of annual temperature fluctuation and a shorter activity season, which favours GSD. This differentiation causes divergent natural selection, allowing for the emergence of sex chromosomes in the highlands.

Spencer & Janzen (2014) provided further support for this model by incubating painted turtles, *Chrysemys picta*, eggs at different sex-yielding temperatures and measuring various characteristics indicative of fitness. Hatchlings from non-biased nests were less energy efficient and grew less than those of the same sex that were incubated in sex-biased producing temperatures, which also showed higher first-year survivorship. Thus, TSD may be advantageous and selected for in turtles, as embryo energy efficiency and hatchling size are optimized for each sex at single-sex incubation temperatures and are indicative of first-year survivorship. This suggests that natural selection would favour TSD, when enhancing the fitness of offspring, or is simply maintaining TSD when previously existing in the phylogeny.

1.2. Impacts of climate change and implication of TSD

Recent shifts in climate regimes at a global level have and are affecting the phenology and survival of many different animal and plant species (Cunze *et al.*, 2013; Thomas *et al.*, 2004; Thuiller *et al.*, 2008). TSD species have undergone various climatic extremes throughout the millennia, which suggests the possibility of adaptation or acclimation. However, contemporary climate change is taking place at such a fast

pace that global warming might pose a threat of biased sex ratios and, therefore, demographic collapse, depending on species' ecological and physiological traits and evolutionary potential - TSD pattern, width of TRT, generation length, genetic diversity, habitat fragmentation, dispersal ability and heritability of TSD traits (Mitchell & Janzen, 2009). Strongly biased sex-ratios can impact the persistence of any species' population (Mitchell & Janzen, 2010), but species with TSD may be particularly vulnerable. Reptiles with TSD may be threatened by climate change because under a warming and fluctuating climate (Stouffer & Wetherald, 2007; Boer, 2009) populations may produce offspring of predominately one sex, disrupting mating systems and therefore leading to decline (Mitchell *et al.*, 2008). However, the severity of the effect probably depends on the mating system and the life history of the target species, with monogamous and/or short living species being more affected, while polygynous or long living ones being able to buffer shifts better.

The effects of rising temperatures are already being observed in various species, like for example sea turtles, of which some populations are undergoing processes of feminization. The loggerhead sea turtle, *Caretta caretta*, is one of these species, with estimated 84% female hatchlings currently being produced in the Republic of Cabo Verde (which holds up to 15% of global nesting by this species); by 2100 or with an increase of ambient temperature of up to 7.5°C (the maximum predicted increase), sex ratios could reach over 99% female or cease to produce males, with many nests incubating at lethally high temperatures (Tanner *et al.*, 2019; Hawkes *et al.*, 2007). The same is expected for the green sea turtle, *Chelonia mydas*; Jensen *et al.* (2018) shows that some populations have been producing primarily females for more than two decades and that complete feminization is possible in the near future. Furthermore, Tomillo *et al.* (2015) showed that, in the leatherback sea turtle, *Dermochelys coriacea*, even though TSD partly mitigates the effects of an increase in mean temperature through the increase of female offspring and, therefore, fecundity, this mechanism will become inefficient as temperatures rise to levels predicted by climate change models. In general, the reaction to climatic changes will depend on the mating system and life history traits of the species. In the case of heritability of the pivotal temperature, the few females producing male hatchlings would have stronger chances of passing the genes to the following generation, balancing the sex-ratio again. Although this could theoretically be fast, turtles have very long generation times and very high hatchling mortality. Another worrisome fact is that climatic shifts might force reptiles to lay eggs in locations that, even if thermally

adequate, could be unsuitable because of conflict with other environmental factors such as humidity and exposure to predators.

While feminization does pose problems when the female to male ratio is high enough that there are not enough males to keep a steady population density, Patricio *et al.* (2018) indicates that the increase in mean temperatures will contribute to population growth in a globally important population of *Chelonia mydas*, with an expected consequential increase in nesting females. However, as the temperatures approach lethally high levels, this population will start to decline. Feminization might not cause any issues in the short term because scarcer males may mate with multiple females but, in a long term perspective, this will produce inbreeding.

Species with the FM TSD pattern are at risk of masculinization, which is a bigger and more immediate threat than feminization due to the quick decrease in fecundation with the lack of reproducing females. The only living species that presents this pattern is the tuatara, *Sphenodon punctatus*. Furthermore, the Brother's Island tuatara, *Sphenodon punctatus guntheri*, also has low genetic diversity, a short width of TRT, ($\leq 1^\circ\text{C}$) a very long generation time (40-50 years), a small clutch size (3-8 eggs every 9 years) and a minute and fragmented habitat (Mitchell & Janzen, 2010; Mitchell *et al.*, 2010). Simulations showed that populations with 75% males could persist for 2000 years, but populations with 85% males would become extinct in 300 years. Recent models have predicted that, by 2085, the male to female ratio would be 100% (Mitchell *et al.*, 2010).

Apart from the increase in mean temperatures that is already recorded and expected to get worse in the future due to climate change, a different threat to TSD species that still remains a bit uncertain is the increased thermal variance. A study carried out in 2011 with *Chrysemys picta*, touched on the subject, indicating that big thermal fluctuations add complexity to the developmental and sexual responses (Neuwald & Valenzuela, 2011). Later, Valenzuela *et al.* (2018) further explored the subject of thermal variance, concluding that broader fluctuations feminize developing embryos around male-producing temperatures and sometimes cause death around female-producing profiles, indicating that, the feminization of TSD turtle populations will accelerate as thermal variance increases with climate change.

1.3. Possible natural mitigation strategies

Theoretically, it is easy to predict that a wider TRT gives a species more resilience towards increasing temperatures. This was demonstrated by Hulin *et al.* (2009) who, with the help of mechanistic models, forecasted that the proportion of nests producing both sexes is higher in species with a wider TRT and that these are more likely to be able to evolve in response to new thermal conditions, which indicates a lower risk of demographic collapse.

Climate change affects nest-building TSD species through suboptimal sex allocation. Many animals are known to be capable of adjusting nesting behaviour adaptively across environmental and seasonal gradients, which may facilitate their ability to respond to ongoing thermal change. These adaptations may result from rapid evolutionary changes such as selection on nesting behaviour, in organisms with shorter generation times (Mainwaring *et al.*, 2017) but can, however, be prevented by phenomena of individual phenotypic plasticity in species with long generation times, acting as an urgency mechanism (Fox *et al.*, 2019) and only when the available thermal range exceeds the limits for nesting is that adaptation takes charge.

Reptiles with TSD may be able to balance sex ratios by nesting earlier in the season, as demonstrated in *Caretta caretta* (Hawkes *et al.*, 2007). A population of painted turtles, *Chrysemys picta*, shows individual plasticity on the date of first nesting each year, depending on the climate from the previous winter. However, sex ratios were much more affected by increases in summer temperature than by earlier egg layings, which suggests that, most likely, the existence plasticity in timing of nesting will not compensate effects of temperature increase. Another study done on nesting lizards, *Bassiana duperreyi*, shows that nesting date shifts will be lacking considering an ambient temperature increase of 4°C over the current century (IPCC, 2013) and predict a sex-ratio bias and egg mortality, regardless of realistic advances in nesting date (Telemeco *et al.*, 2009). Even though changes in nesting phenology facilitate adaptation to climate change, they seem insufficient to prevent serious consequences in species with TSD.

The choice of nest-site location and design also provide means of adaptation in which rapid changes can occur within the individual lifetimes, with the creation of suitable microhabitats that balance sex ratios. Animals breeding in hot environments choose cooler sites over time (Telemeco *et al.*, 2009, 2013) and ecological and evolutionary shifts are possible (Refsnider & Janzen, 2010). A study done on Australian

water dragons, *Intellagama lesueurii*, showed that these lizards compensate for climatic differences by selecting open nesting spots with higher radiation at colder locations. On the other hand, Refsnider & Janzen (2012) suggests that females of *Chrysemys picta* from different populations choose nest sites with different shade cover and that this might be a behaviorally plastic mechanism that allows compensation for change in temperatures during embryonic development. Adding to this, species with large clutch sizes may even experience different incubation conditions in different parts of the nest, with eggs in the interior suffering less fluctuations, which would possibly serve as a buffer, while species with small, fixed clutch sizes do not have that possibility.

A different mitigation strategy could be for TSD species to change their distribution. In fact, dispersal greatly influences range shift in TSD species, more than in GSD species. According to Boyle et al. (2016), range shifts may be influenced by biased sex ratios, with the mixing of the rare sex (females) with males located at the colder edges of the range, as both sexes disperse. There are also signs of a peripheral, northwards expansion in *Tarentola mauritanica* populations from SE France (Geniez & Cheylan, 2012), although it seems that the species is using phenotypic plasticity to cope with climatic variation, becoming more diurnal and more associated with human buildings in the north than in the south, where its nocturnality is maintained. However, in the event of a 3°C warming over the next 100 years, even TSD species will have a limited capacity for dispersal followed by range shift (Boyle et al., 2016).

2. Symmetry as measure for developmental stability

Developmental instability is the tendency of small perturbations in the developmental environment to produce morphological deviations from standard development given the incapability of the embryo to correct them. One of those deviations is, for instance, organ asymmetry. In bilateral organisms, subtle departures from symmetry are represented by frequency distributions of right trait measure minus left trait measure ($R - L$) (Palmer, 1994). Such distributions are commonly divided into three types of asymmetry: fluctuating asymmetry (FA), usually defined as small and random deviations in the symmetry of bilateral characters of organisms, with a mean equal to zero and variation normally distributed around that mean; directional

asymmetry (DA), which occurs when there is a greater development of a character at one side of the body plan of symmetry than at the other, with most individuals being asymmetrical in the same direction; and antisymmetry, which occurs when one side is consistently larger than the other, but the larger side is randomly distributed among individuals (Palmer, 1994). FA is inversely related to developmental stability (DS) and is at its lowest under optimal developmental conditions (figure 4). Due to its probabilistic nature, FA is a property of the population rather than of the individual, which may be the result of environmental perturbations (e.g. temperature, pollution, subnutrition), to limited capability of the embryo to correct it (e.g. inbreeding) or to both, and which must be zero-centred and normally distributed.

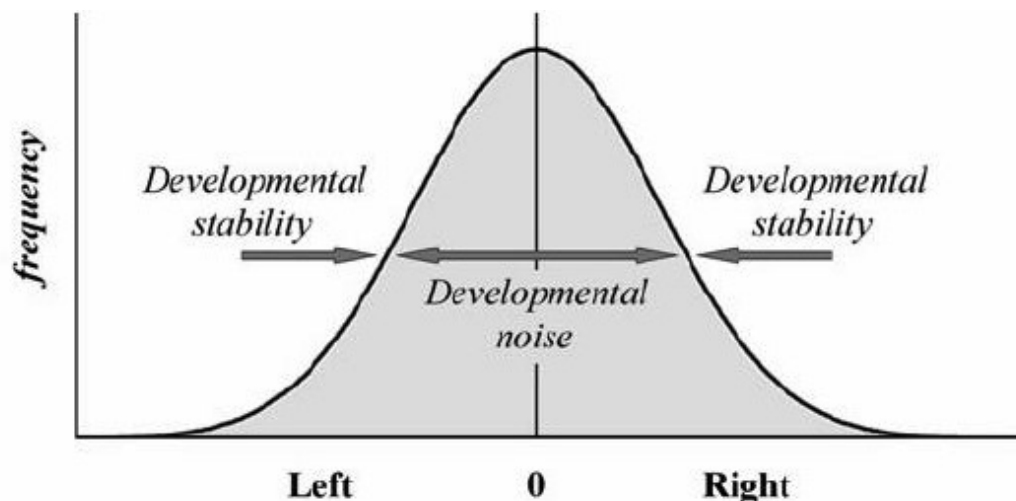


Figure 4 - The scale of fluctuating asymmetry demonstrates the interaction between two opposing processes: developmental noise and developmental stability (modified from Palmer, 1994) (Kegley & Hemingway, 2005).

A study combining measurements in adults and incubation experiments performed on the sand lizard, *Lacerta agilis*, showed that optimum incubation temperatures had the same effect across different populations, with the relationship between FA and incubation temperature having a U-shaped curve, with the minimum of the curve corresponding to the optimum temperature; understandably, DS was higher at the central part of the curve, decreasing towards the periphery, therefore indicating a dependence of DS from incubation temperature (Zhdanova & Zakharov, 2006). Similarly, Qualls & Andrews (1999) experimentally incubated eggs of the striped plateau lizard, *Sceloporus virgatus*, at a range of temperatures and found that colder

nest temperatures induced greater FA in hatchlings. They also found that lower temperatures slowed embryonic development and decreased hatching success. On the contrary, in laboratory experiments with eggs of the oriental garden lizard, *Calotes versicolor*, Ji *et al.* (2002) showed that the highest incubation temperatures produced the highest levels of FA. Both studies show that lizards incubated at extreme temperatures show higher levels of FA and also that more asymmetric hatchlings have lower fitness. Braña & Ji (2000) also found a strong relationship between thermal stress and fitness on the hatchlings of wall lizards, *Podarcis muralis*. However, they did not report any clear trend on fluctuating asymmetry and temperature interaction. In snakes, Arnold & Peterson (2002) showed that asymmetries (not specified) of the neonates are related to extreme incubation temperatures in the viviparous garter snake, *Thamnophis elegans*. Löwenborg *et al.* (2011) identified a relationship between FA and incubation temperature in the oviparous grass snake, *Natrix natrix*. Eggs of this species incubated on lower temperatures had a higher tendency to generate asymmetric individuals. They also found lower fitness in snakes incubated at low temperatures.

3. *Tarentola mauritanica*

The commonly named Moorish gecko, *Tarentola mauritanica*, from the family Phyllodactylidae, is native to the western Mediterranean area of Africa and Europe (Vogrin *et al.*, 2017). In the Iberian Peninsula, it is mostly found in the centre, south and east, having been cited in various locations of the northwest (Hódar, 2002; Cabana & López-Bao, 2007; Paramos & Ayres, 2007; Cabana, 2008; Gosá *et al.*, 2011; Tejado & Potes, 2011; Salvador, 2014). There are populations distributed across most of continental Portugal (Godinho *et al.*, 1999; Malkmus, 2004; Perera *et al.*, 2008; Pires Ceriaco & Pimentel Marques, 2011), with the northernmost population found in Bragança district (Malkmus, 1996). It is found in various different habitats like natural rocky areas and cliffs, but it is generally associated with humanized structures such as stone walls, ruins, building walls and inside houses; it is not usually present in forested areas although animals can be found climbing trees (Vogrin *et al.*, 2017). The females lay various clutches of one to two eggs per reproductive season; these may be laid communally, typically under stones, in cracks and in hollow trees (Vogrin *et al.*, 2017). This gecko is recognized as a species-complex encompassing six genetic lineages, considered as putative distinct species; the European/North African clade is the one

with the largest geographic distribution, occupying most of the western Mediterranean Basin and, therefore, a series of distinct environmental conditions (Figure 5; Rato *et al.*, 2015; Rato *et al.*, 2016; Harris *et al.*, 2004a, Harris *et al.*, 2004b, Rato *et al.*, 2012). Rato *et al.* (2015) demonstrated that the divergence in the ecological models for these clades results from distinct tolerances to humidity compared to the remaining lineages. Support for these conclusions, at least in the Iberian Peninsula, is provided in Rato & Carretero (2015), with the adult geckos from the European lineage presenting a higher and more diverse pattern of water loss among populations when compared with the Iberian lineage.

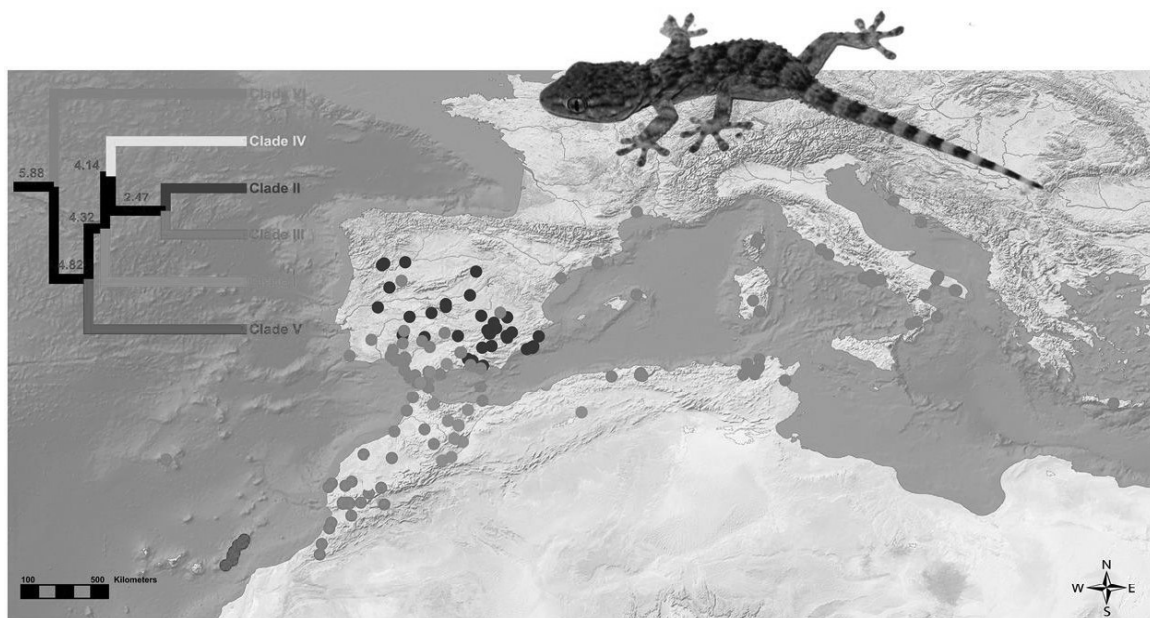


Figure 5 - Map illustrating the occurrence records of all six clades of *Tarentola mauritanica* and a calibrated phylogenetic tree with node ages (in Ma), modified from Rato *et al.* (2012) (Rato *et al.*, 2015).

Remarkably, the only Squamata in the Mediterranean Basin presenting TDS are within the genus *Tarentola* and two studies on sex determination of this genus suggest a TSD MF pattern for *T. mauritanica* (Hielen, 1992; Nettmann & Rykena, 1985), with Hielen (1992) suggesting a pivotal temperature of 26.8°C with experiments performed on animals from the Balearic island Menorca, belonging to the European clade. Moreover, none of the karyotyped species of this genus show evidence of heteromorphic sex chromosomes (Odierna, 1994; De Smet, 1981), which suggests that this species has TSD. Although there is already some evidence about the presence of TSD in *T. mauritanica* and other congeneric species, there are no studies about the

adaptation of TSD components such as nest-choice, sex-ratio and pivotal change to local conditions. This information is crucial to determine the response of this species to the inevitable climate and environmental changes that will inevitably happen in the near future.

3.1. *Tarentola mauritanica* as model species

This gecko is a fitting model species for this research for many reasons. From a scientific point of view, it is, apart from turtles and terrapins (for which also the knowledge on TSD patterns is scarce), the only lizard of the entire European continent presenting this type of sex determination. Furthermore, because of this physiological trait, its sex-ratio is highly sensitive to shifts in incubation temperatures and therefore, to the possible effects of climate change, which can be seen after only one generation: *T. mauritanica* lay various clutches of two, rarely one, egg, with a short generation time, opposite life-history traits of turtles or crocodiles. Due to this small clutch size, eggs of the same clutch should experience very similar incubation conditions (eg. temperature, humidity) both in nature or in the lab, as opposed to almost all other TSD species analysed to date. Another reason is that, being such a widespread species, *T. mauritanica* is subject to a variety of environmental regimes, which allows for the study of patterns of adaptation and phenotypic plasticity. From a scientifically informed conservation and management biology point of view, it is important to assess if and how these animals will be affected by and respond to the predicted inevitable changes in climate, since this might have implications to biodiversity, agriculture and public health. Geckos are generally recognized to play an important role in the control of many pests, including mosquitos, which can be vectors of serious diseases affecting human populations, such as yellow fever, dengue and skin necrosis (Canyon & Hii, 1997, Ramires & Fraguas, 2004). *Tarentola mauritanica* is known to consume flying insects including mosquitoes in both natural and urbanized habitats (Gil *et al.*, 1994; Hódar *et al.*, 2006), tending to eat more in the second case. As part of the biodiversity of Portuguese agricultural systems, *T. mauritanica* serve not only as bioindicators but also help in the fight against pests, improving the quality and production of agricultural crops (Simões, 2007; Ferreira *et al.*, 2009). For these reasons, it is important to assess if and how these animals will respond to climate change, since this might have implications to biodiversity, agriculture and public health.

4. Research objectives

Using as model species the putative TSD gecko *Tarentola mauritanica*, the three main objectives of the research performed in this MSc dissertation are to:

- confirm the existence of TSD in this species;
 - if true, describing the pattern of sex determination;
- evaluate the existence of adaptation of key behavioral and physiological TSD components, namely female nest-site choice and pivotal temperature, to local environments (these will be compared among populations at different latitudes in order to demonstrate the existence of behavioural plasticity and/or physiological adaptation as ways to maintain balanced sex-ratios in a widely distributed species);
- evaluate the existence of different patterns of FA at the population level as an indication of environmental disturbance;
 - assess the connection between this pattern and incubation temperature or, on the other hand, heritability, while also making inferences about optimal conditions and individual fitness.

In the future, this research will continue aiming at determining the way different predicted climatic scenarios could affect the population dynamics and the survival of the Moorish gecko and discovering the candidate genes involved in TSD in this species through gene expression levels obtained by NGS transcriptome assembly.

The expected results for the first objective are the confirmation of TSD in *T. mauritanica* and a MF pattern with a pivotal temperature preliminary estimated at around 26.8°C (Hielen, 1992, Nettmann & Rykena, 1985).

As for the second objective, it is expected that populations across the species' geographic range will either present a fixed temperature selection - phylogenetic inertia - or a counter gradient selection to compensate thermal variation - behavioral plasticity or local adaptation. This second result makes sense if factors contributing to incubation regime are driven primarily by adaptation for successful embryonic development and survival, and less by selection on sex-ratio (Ewert *et al.*, 2005; Refsnider *et al.*, 2014; Refsnider & Janzen, 2016; Schwarzkopf & Brooks, 1987). For this reason, females of southern populations may choose nest sites that experience relatively cool incubation

conditions in order to reduce the likelihood that embryos undergo lethally high temperatures and, contrarily, females up north may prefer relatively warm incubation conditions so that it is less probable that embryos fail to develop at lower temperatures. Based on previous fieldwork expeditions, adult sex-ratios of each population are not expected to be strongly biased.

Regarding the conservation consequences, TSD and its components have been under research since the 70's (Bull, 1980; Bull & Vogt, 1981; Charnov & Bull, 1977) and acquiring and increasing knowledge about it, it's crucial in order to apply the correct management measures to all TSD species and, in particular, *Tarentola mauritanica*. These species are evidently particularly affected by thermal variation and, depending on the existence or absence of local and populational adaptations, vulnerable to the future anticipated climatic changes. The mean global temperature has risen by 0.85 °C since the Industrial Revolution (Intergovernmental Panel on Climate Change, 2013). 33–50% of the Earth's land surface has been altered by humans and even if human populations were to decline substantially or use of land become more efficient, the current human transformation of ecosystems have already irreversibly altered the terrestrial biosphere enough to leave a geological record differing substantially from that of the Holocene or any prior epoch (Ellis, 2011; Vitousek *et al.*, 1997). The speed at which anthropogenic environmental changes are occurring may outpace many organisms' ability to adapt through evolutionary responses (Lavergne *et al.*, 2010). *Tarentola mauritanica* might be benefited by climate change, indicated by its current expansion in distribution towards the north and higher altitudes, by their partial nocturnality and low water dependence in adults and eggs, enabling them to inhabit warmer and drier habitats than other reptile species, taking therefore advantage of warming and aridification. TSD is not necessarily involved in this adaptability to warmer and more arid locations; it is possible that fast TSD acclimation or adaptation could also be of contribution but it must be demonstrated experimentally.

Regarding the last objective of this thesis, although the number of lamellae is likely heritable, just like scales and shape (Üveges *et al.*, 2012; Sacchi *et al.*, 2016), FA is mainly an indicator of environmental disturbance (considering the absence of inbreeding), which could be different for both sexes if, as commonly admitted, TSD arises due to different optimal temperatures for males and females. It is expected that wild populations from locations that experience colder, male-producing mean temperatures will have low FA in males and high FA in females and the opposite is expected from populations that experience warmer, female-producing mean

temperatures. The effects of thermal variance are still unknown. As for the artificially incubated individuals, these are expected to be more symmetrical as the incubation temperature approaches optimal levels for the production of each sex.

Materials and methodology

1. Sampling

This study was performed using four Iberian populations, collected during spring 2019, in Évora (38.560533, -7.912956), Torres Vedras (39.089592, -9.254778), Portimão (37.286833, -8.551722) and Ayamonte (37.204126, -6.933142) (Figure 6). In order to avoid the detection of differences due to phylogeny, all populations belong to the European clade of the *Tarentola mauritanica* species-complex, already identified by molecular markers (Rato *et al.*, 2016). During fieldwork, a minimum of 20 animals of each sex were collected (giving preference to natural wild couples) and transported to CIBIO-InBIO'S thermal ecophysiological lab facilities. Animals were randomly caught using a noose (following García-Muñoz & Sillero, 2010) or by hand, frequently attracted by a laser pointer (Cole, 2004). All captures were performed with the necessary permits requested to national authorities of both Portugal and Spain.



Figure 6 - Map with the sampling locations for each of the four populations: Torres Vedras in pink, Évora in orange, Portimão in green and Ayamonte in blue.

2. Monitoring local climatic conditions

Considering that in nature, *Tarentola* geckos lay their eggs under rocks, tree branches and pieces of wood or bury them in the soil (González de la Vega, 1988), a few possible nests were identified in the field and an iButton datalogger (i-button DS1922L, precision (0.001°C) and accuracy (0.001%)) was placed in each of them in order to record temperature oscillations throughout the reproductive season (April to August). This way, for each nest within each population, data on average nest temperature as well as the daily variance will be obtained.

3. Husbandry

The animals were kept under controlled temperature conditions, at around 18°C-20°C room temperature and routinely heated with 150W infrared light bulbs, in 50x30x25cm terraria with securely stacked rocks, which serve as refugia places, and a water dish. In order to maximize reproduction with food availability, the animals were fed every day with live house crickets (*Achetus domesticus*) and mealworm larvae (*Tenebrio molitor*) powdered with vitamin and calcium supplements. Although the clutch size per female is 1-2 eggs, female Moorish geckos can lay multiple clutches throughout the reproductive season; in the field they lay 1-3 clutches but in captivity up to 7 clutches have been reported (Martínez-Rica, 1974). The adult couples from each of the four populations were kept together during the reproductive season so that several clutches were guaranteed throughout the experiment, increasing considerably the sample size.

4. Oviposition temperature choice

Females were examined regularly for signs of advanced egg development. Spotted pregnant females were individually exposed to a thermal gradient ranging from 22°C to 45°C, in a 100x30x40cm Plexiglas terrarium with a 150W infrared bulb fixed 15cm above the substrate and maintaining a natural photoperiod (Rato & Carretero, 2015; Carretero, 2008), in order to assess the temperature conditions at which females prefer to lay their eggs. The results from this part of the experiment, when compared among all populations of *T. mauritanica*, will give us information about the existence of

any match between female behaviour and population environmental conditions, hence, if there is or not adaptation to their local climate.

5. Sex ratio and pivotal temperature under constant incubation temperature

Immediately after oviposition, each egg was placed over vermiculite and in one of six egg incubators (RCOM Juragon Pro), previously set to constant temperatures ranging from 25°C to 30°C, always at 90% humidity. This part of the study consists in recording the hatchlings' sex when their development is subjected to a constant incubation temperature. In order to avoid pseudoreplication at the clutch level as well as to disentangle between maternal and temperature effects on phenotypic sex, eggs from the same clutch were incubated at distinct temperatures, unless they could not be physically separated without risking the integrity of the egg. After hatching, sex identification of the juveniles was performed by gonadal inspection with the help of an electronic magnifier, after being sacrificed following the "cooling then freezing" protocol from (Shine *et al.*, 2015), demonstrated to be the less painful way to euthanize reptiles. Hatchling sex-ratio and embryo mortality was registered. These results complete the main objective, providing knowledge on which sex is determined at the different constant temperatures and, therefore, the pivotal temperature for each different population: this crucial TSD component can vary between species' populations as a form of adaptation to local climatic conditions.

6. Asymmetry

Immediately after hatching, the snout-vent length of each juvenile was measured to the closest 0.01 mm using a digital calliper. Also, photos of the lamellae of the fifth finger (LFF) were taken on both sides of the body. The same procedure was also performed for the mothers (and putative fathers, although paternity was not genetically tested and pregnancy of females due to sperm retention from copulations in the field cannot be completely discarded), in order to disentangle between temperature and heritability effects. The LFF meristic trait is not only important for the taxonomy of *Tarentola* (e.g. Bshaena & Joger, 2013), but it was chosen mainly due to its strong functional role in gecko's locomotion (Gamble *et al.*, 2012); predictions are that certain

incubating temperatures should be optimal for each sex, leading to less asymmetrical and more fitted individuals. The number of lamellae (~15-20) is large enough to adjust to a normal and minimize threshold effects (Soulé, 1967). For each individual, the number of LFF were recorded twice from the digital photos available, allowing several days of rest between the first and the second counting and randomizing the order of examined specimens, to ensure the independence of trait counts.

7. Data analysis

Once all the data was collected, all plots, descriptive statistical analyses, general linear models and model selections were performed using the software Statistica v.13 (TIBCO Software Inc., 2018). Generalized linear model (GLM) building was performed to know which variables best explained oviposition temperature; GLM were used to infer effects of incubation temperature on hatchling sex; female weight on hatchling weight; population, oviposition date, weight and SVL on oviposition temperature; weight and SVL and interactions on total number of ovulations; female FA and incubation temperature and interactions on hatchling FA. A logistic regression was made for the visualization of the male/female hatchling yield and to find out the pivotal temperature. A t-test was performed in order to find out significant differences between populations' asymmetry levels. Histograms, box plots and scatterplots were created in order to represent the data and the performed analyses.

Results

1. Sex-ratio and pivotal temperature

Throughout the reproductive season, from the 80 (20 by population) females kept captive, we were able to incubate a total of 60 eggs. Of those, only 28 successfully developed and hatched. A histogram comparing the mortality rate of the hatchlings at different incubation temperatures (Figure 7) shows that the biggest viability was recorded at the incubation temperature of 28°C, with 67.9% of the incubated eggs successfully hatching. The mortality was the highest when eggs were incubated at 25°C, at which 81.8% off incubated eggs never hatched. 33.3% of eggs incubated at 30°C resulted in viable hatchlings.

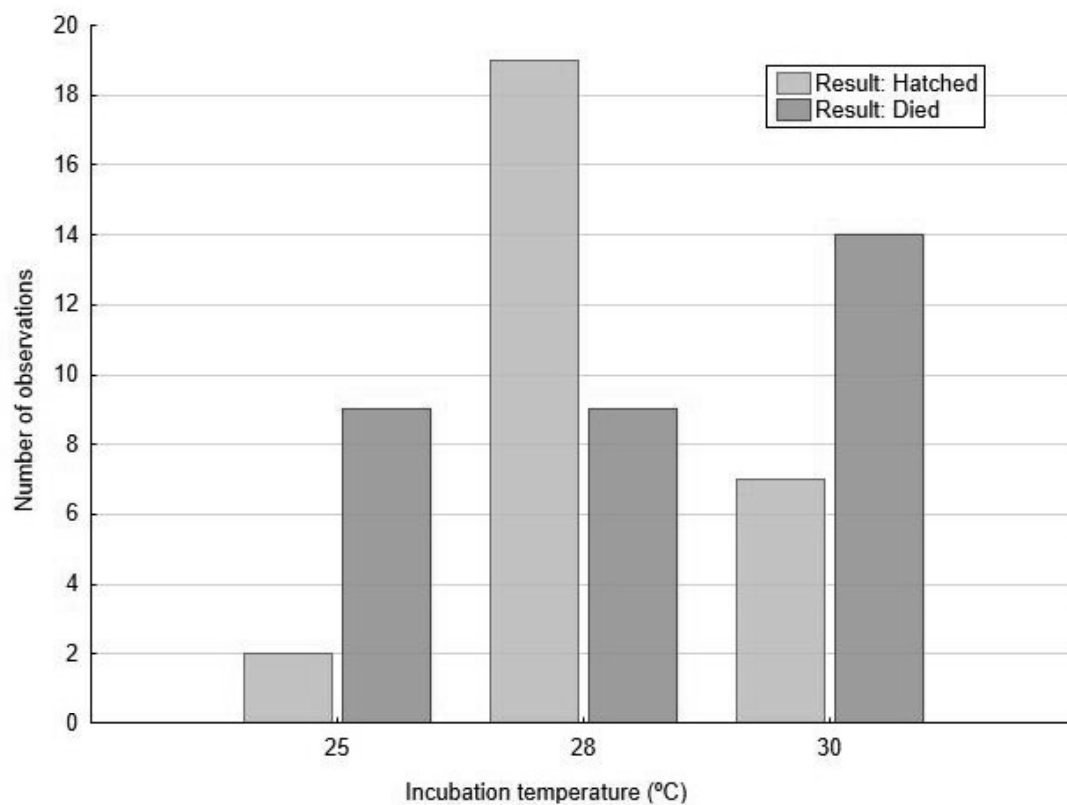


Figure 7 - Frequency of the result of incubation, categorized by each incubation temperature (in °C).

As to the first objective of this project, the sex-ratio at different incubation temperatures, as seen the histogram with the frequency of both sexes (Figure 8),

shows a separation in the yield of male hatchlings between higher and lower temperatures. Eggs incubated at 30°C solely resulted in females; 94.4% of eggs incubated at 28°C resulted in females but eggs incubated at 25°C yielded only males. However, due to the high mortality rate, there is a lack of data for the two extreme temperatures.

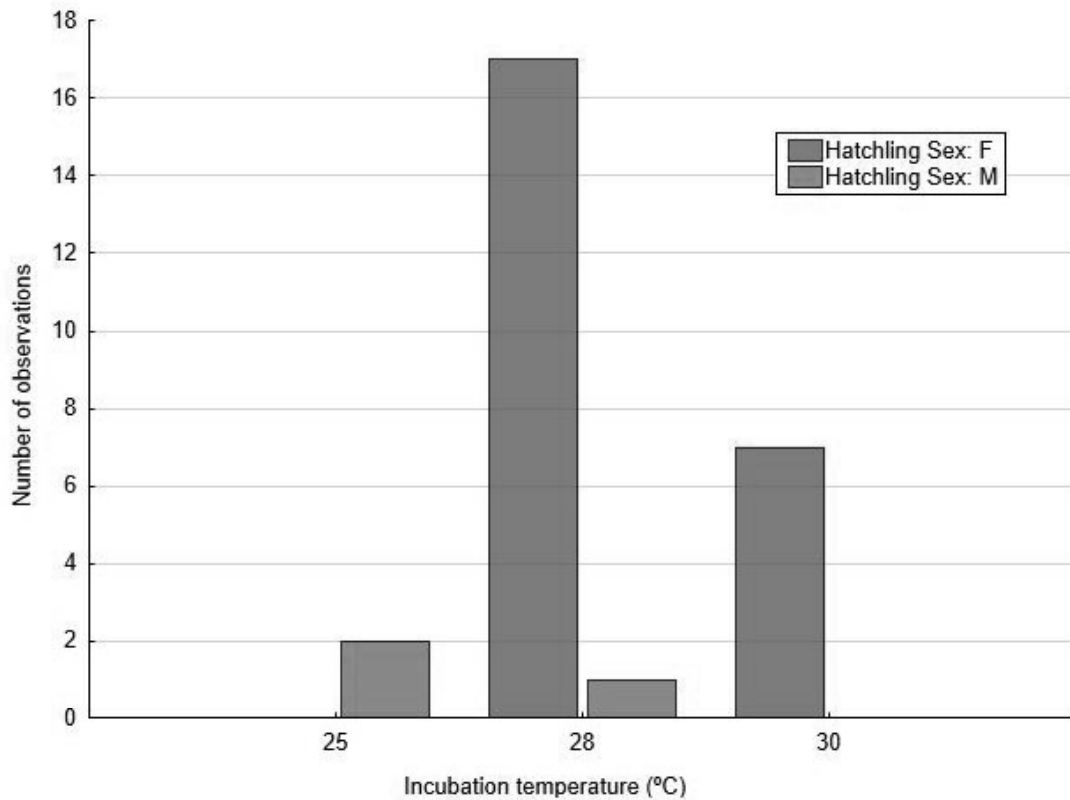


Figure 8 - Frequency of the different hatchling sexes, categorized by each incubation temperature (in °C).

Significance tests for a GLM with a p-value < 0.0001 indicate that incubation temperature greatly influences the sex of the hatchlings (Table 1). With the help of a logistic regression line, despite the lack of data for the temperatures of 26, 27 and 29°C, the pivotal temperature seems to be located at 27.78°C, at which both females and males hatch with a frequency of exactly 50% (Figure 9).

Table 1 - Univariate Tests of Significance for the GLM of hatchling sex with the incubation temperature variable. Sigma-restricted parameterization. Effective hypothesis decomposition; Std. Error of Estimate: 0.1984.

Effects	SS	df	MS	F	p
Intercept	1.59	1	1.59	40,54	0,000001
Female weight	1.72	2	0,86	21,88	0,000004
Error	0.944	24	0.039		

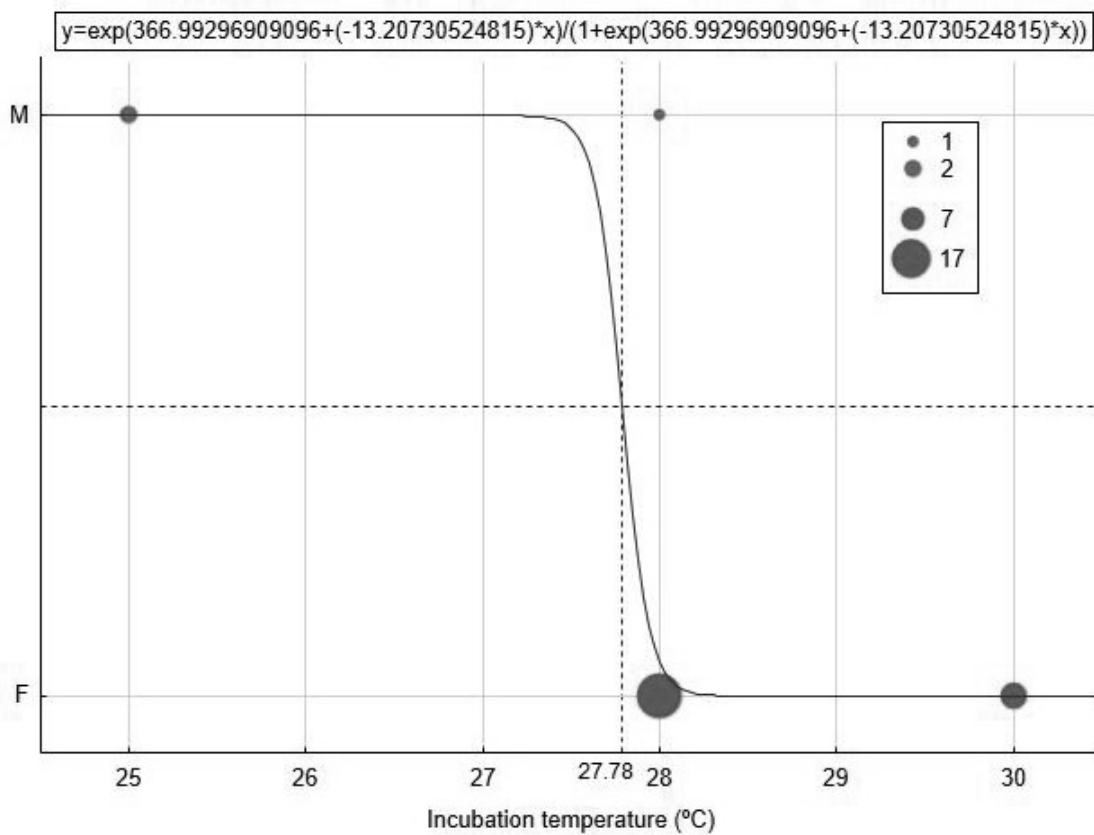


Figure 9 - Frequency of female/male yield against incubation temperature (in °C), with a logistic regression line of male yield. Pivotal temperature ~ 27.78°C.

In Figure 10 we can see that embryo development occurs quicker the higher the incubation temperature is; a Kruskal-Wallis test showed a H statistic of 18.28 and a p-value of 0.0001 indicating statistically significant relation between total incubation days and incubation temperature. There is no clear relation between hatchling weight and incubation temperature, with a Kruskal-Wallis test showed a H statistic of 1.20 and a p-value of 0.55, indicating no evidence of a statistically significant difference between these two variables (Figure 11).

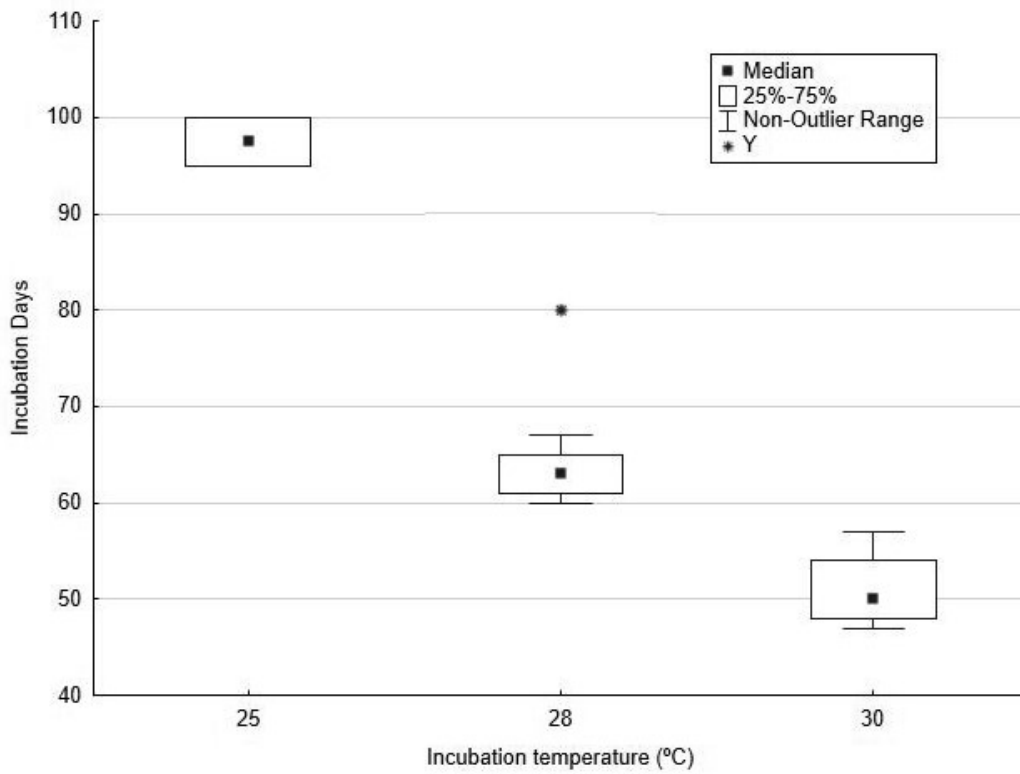


Figure 10 - Total incubation days categorized by each incubation temperature (in °C).

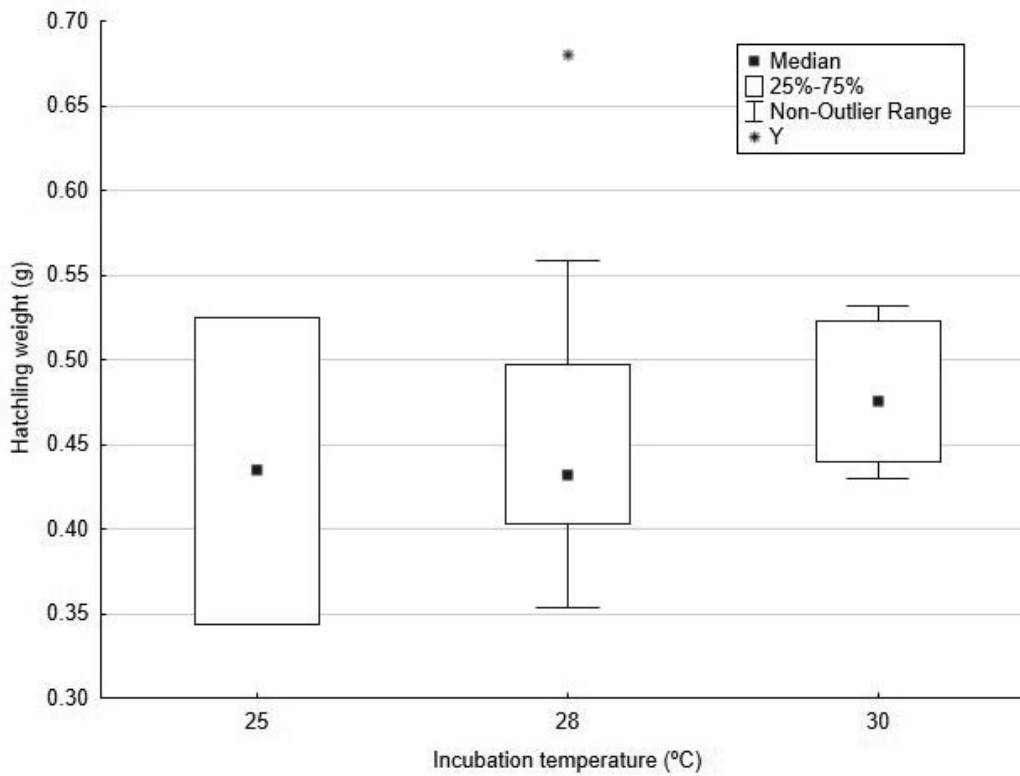


Figure 11 - Hatchling weight (in g) categorized by each incubation temperature (in °C).

As depicted in Table 2, the significance tests for the GLM indicate that female weight explains 23.67% (r^2) of the hatchling weight, and the p-value of 0.018 indicates a small positive correlation between the two variables, with the scatter plot (Figure 12) showing a slope of 0.036.

Table 2 - Univariate Tests of Significance for the GLM of hatchling weight with the female weight variable. Sigma-restricted parameterization. Effective hypothesis decomposition; Std. Error of Estimate: 0.0674.

Effects	SS	df	MS	F	p
Intercept	0.04	1	0.04	9.98	0.0047
Female weight	0.03	1	0.03	6.51	0.0186
Error	0.09	21	0.005		

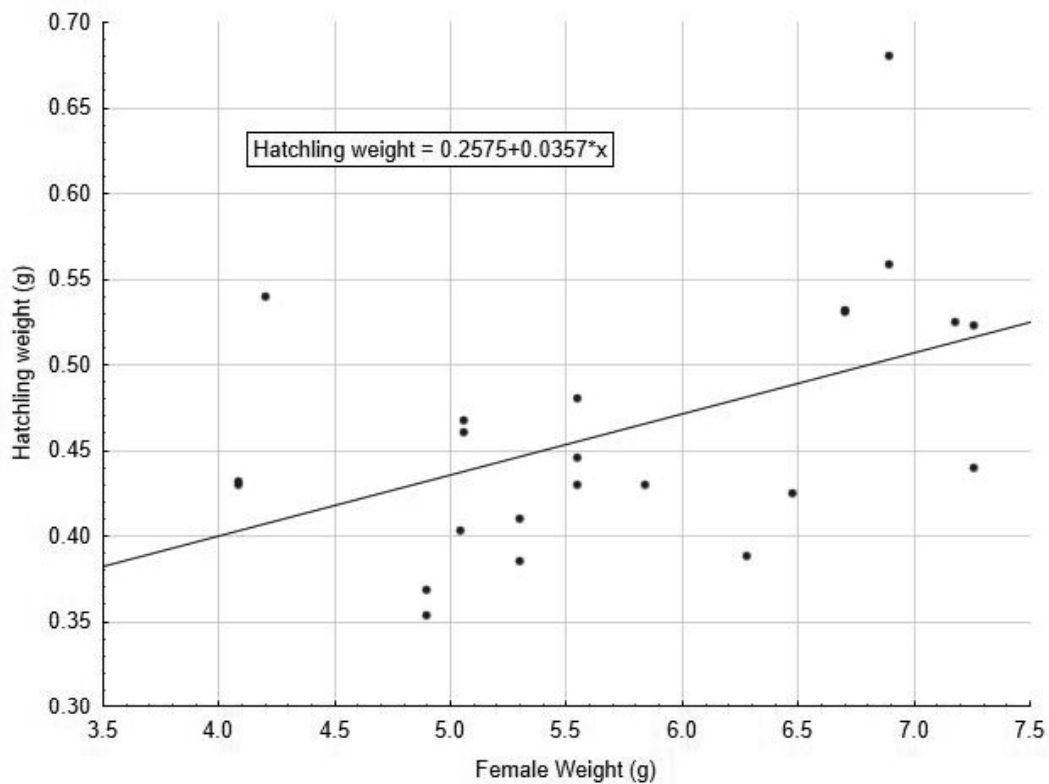


Figure 12 - Hatchling weight compared to mother weight (both in °C) p-value = 0.0186; $r^2 = 0.2367$.

2. Oviposition temperature choice

Through model selection of normal log linear models, with the Akaike Information Criterion (AIC), considering the variables oviposition date, female weight, female SVL and population (Table 3), the simplest model that best describes the data included solely oviposition date, with an AIC of 134.06, followed shortly by a model with only the female weight, with an AIC of 134.89. The model containing only the population presented an AIC of 135.99; however, it should still be informative, because the value of ΔAIC is not bigger than two (1.94; Burnham, 2010)

Table 3 - Model building results for oviposition temperature. Normal distribution; Log function.

Variable 1	Variable 2	Variable 3	Variable 4	df	AIC	ΔAIC (AIC _i -AIC ₁)	L. Ratio Chi ²	p
Oviposition date				1	134.06	0	1.71	0.19
Weight				1	134.89	0.82	0.88	0.35
SVL				1	135.27	1.21	0.50	0.48
Weight	Oviposition date			2	135.31	1.25	2.45	0.29
SVL	Oviposition date			2	135.61	1.55	2.16	0.34
Oviposition date	Population			4	135.69	1.63	6.08	0.19
Population				3	135.99	1.94	3.77	0.29
Weight	SVL			2	136.88	2.82	0.88	0.64
Weight	Oviposition date	Population		5	137.11	3.05	6.66	0.25
SVL	Oviposition date	Population		5	137.16	3.10	6.61	0.25
SVL	Population			4	137.21	3.15	4.56	0.33
Weight	SVL	Oviposition date		3	137.31	3.25	2.46	0.48
Weight	Population			4	137.49	3.43	4.28	0.37
Weight	SVL	Oviposition date	Population	6	139.08	5.02	6.69	0.35
Weight	SVL	Population		5	139.21	5.15	4.56	0.47

In order to test the main hypothesis of local adaptation for nest thermal choice, GLM of the oviposition temperature with the population variable were performed and failed to find significant results (Table 4). A Kruskal-Wallis test rendered a non-significant p-value of 0.28. The box plot of the different oviposition temperatures of each population shows that females from Ayamonte chose the highest temperatures to lay their eggs, while females from Portimão chose the lowest, with Torres Vedras somewhat in the middle; Évora shows the broadest range of temperatures of all populations (Figure 13).

Table 4 - Univariate Tests of Significance for the GLM of oviposition temperature with the population variable. Sigma-restricted parameterization. Effective hypothesis decomposition; Std. Error of Estimate: 3.0855.

Effects	SS	df	MS	F	p
Intercept	26776.90	1	26776.90	2812.53	0.000000
Population	29.90	3	9.97	1.05	0.39
Error	276.10	29	9.52		

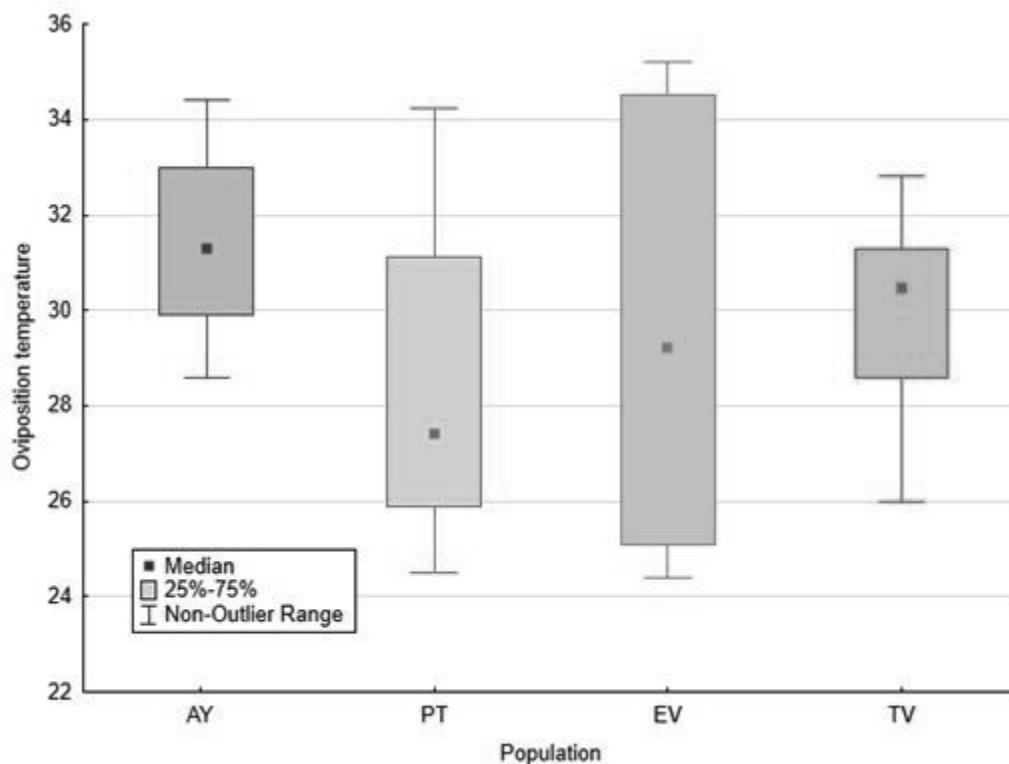


Figure 13 - Oviposition temperature (in °C) chosen by females, categorized by each population.

The results of the GLM of oviposition temperature with the oviposition date variable (Table 5) indicate that this variable is, although close to, not significant in the explanation of the temperatures chosen by the females to lay their eggs, with an r value of 0.30, when populations are pooled. The scatterplot shows a trend for a slightly positive correlation between these two variables, with a slope of 0.039 (Figure 14).

Table 5 - Univariate Tests of Significance for the GLM of oviposition temperature with the oviposition date variable. Sigma-restricted parameterization, Effective hypothesis decomposition; Std. Error of Estimate: 2.9901.

Effects	SS	df	MS	F	p
Intercept	5094.852	1	5094.85	569.85	0.000000
Oviposition date	28.84	1	28.84	3.22	0.08
Error	277.16	31	8.94		

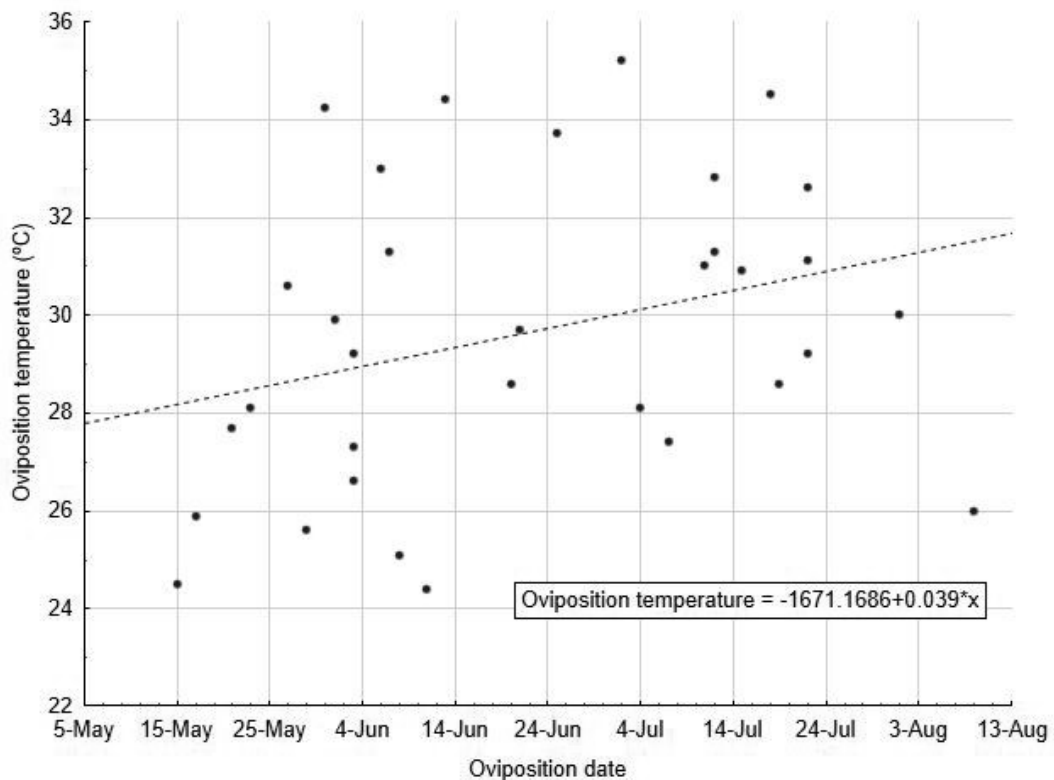


Figure 14 - Oviposition temperature (in °C) chosen by females at different dates, with pooled populations.

However, when categorized by the different populations, as seen in the scatterplot in Figure 15, that trend is lost, indicating either that this trend is false or that there is a lack of sample size.

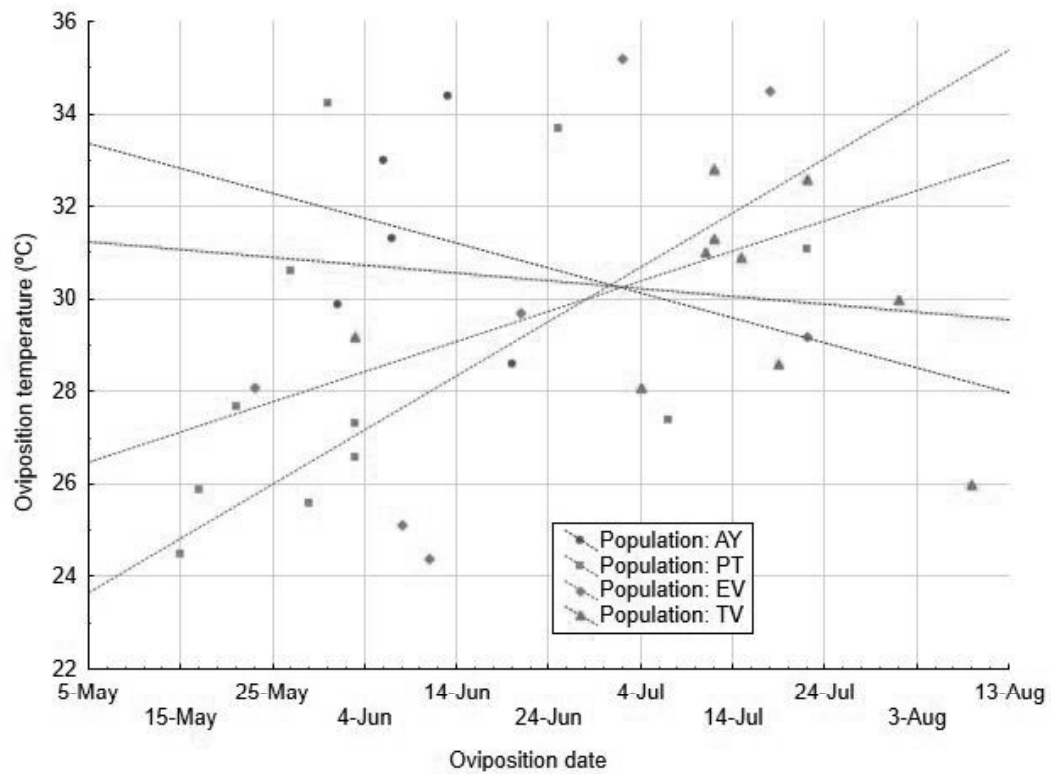


Figure 15 - Oviposition temperature (in °C) chosen by females at different dates, categorized by each population.

Population: AY Oviposition temperature = $2380.5 - 0.0538 * x$

Population: PT Oviposition temperature = $-2824.192 + 0.0654 * x$

Population: EV Oviposition temperature = $-5091.5404 + 0.1173 * x$

Population: TV Oviposition temperature = $766.2834 - 0.0169 * x$

In Tables 6 and 7 are presented the results of significance tests for GLM of oviposition temperature of the variable weight and SVL, respectively. None of these variables were correlated with the oviposition temperature, with r values of -0.18 and -0.07.

Table 6 - Univariate Tests of Significance for GLM of oviposition temperature with the weight variable, Sigma-restricted parameterization. Effective hypothesis decomposition; Std. Error of Estimate: 3.3225.

Effects	SS	df	MS	F	p
Intercept	986.79	1	986.79	89.39	0.000000
Weight	9.03	1	9.03	0.82	0.37
Error	253.89	23	11.04		

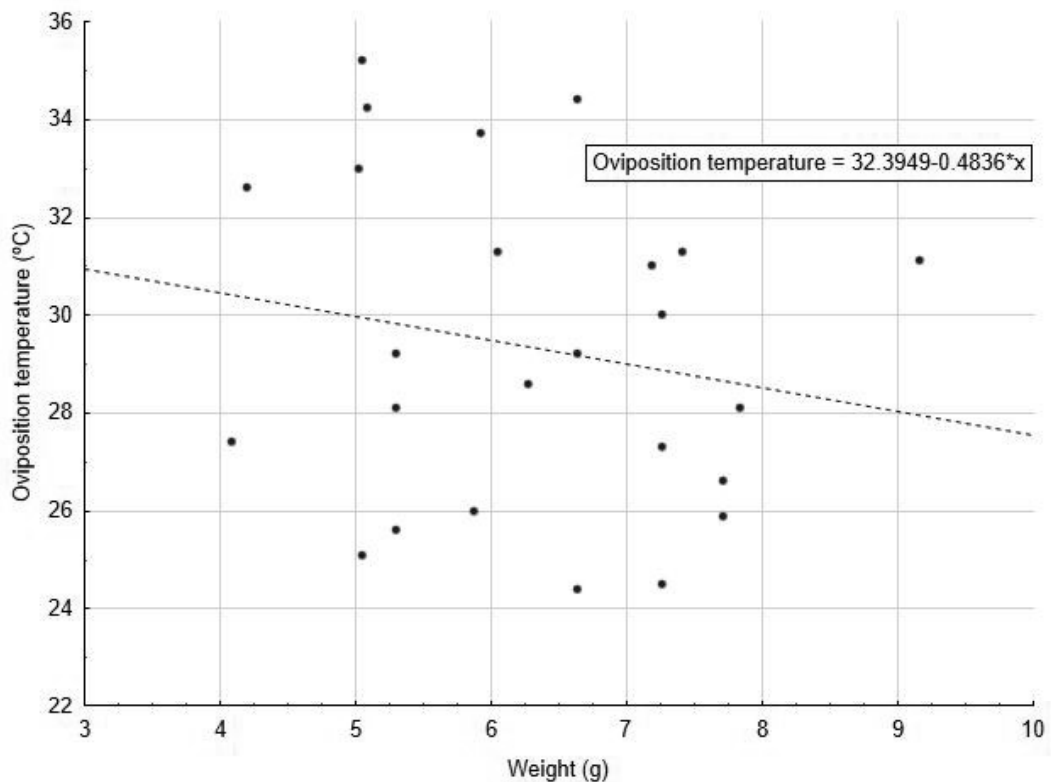


Figure 16 - Oviposition temperature (in °C) chosen by females of different weights (in g).

Table 7 - Univariate Tests of Significance for GLM of oviposition temperature with the weight variable. Sigma-restricted parameterization. Effective hypothesis decomposition; Std. Error of Estimate: 3.3225.

Effects	SS	df	MS	F	p
Intercept	296.52	1	296.52	29.23	0.000007
SVL	1.58	1	1.58	0.16	0.69
Error	304.35	30	10.14		

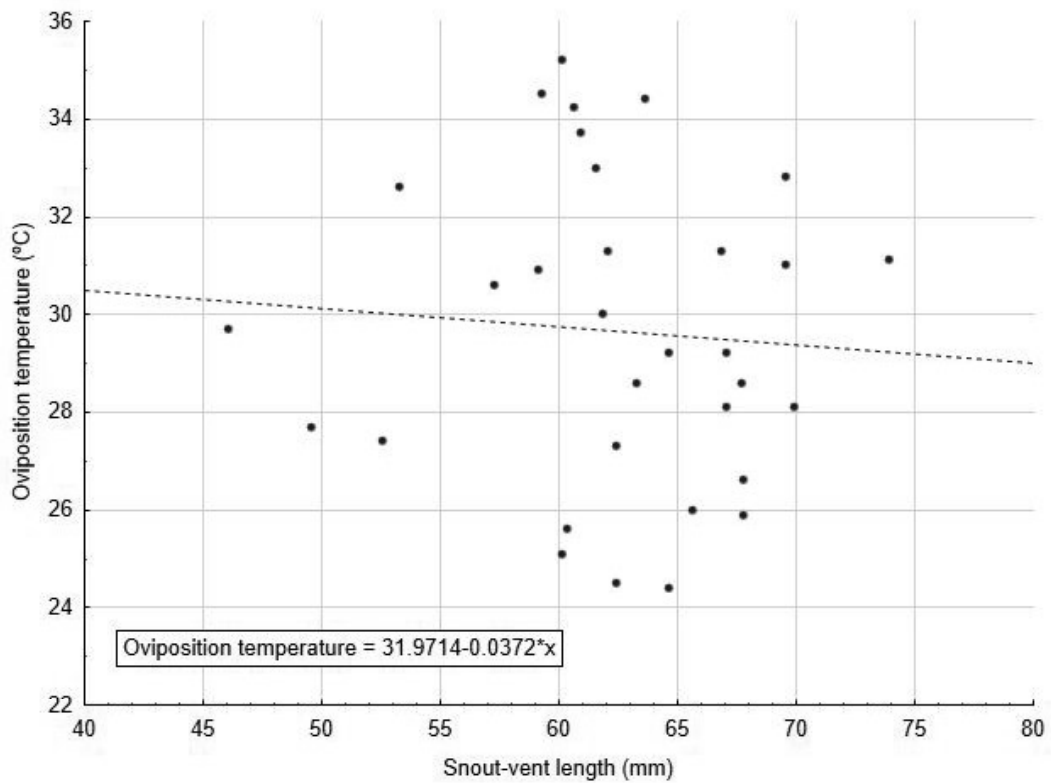


Figure 17 - Oviposition temperature (in °C) chosen by differently sized females (in mm).

3. Female fecundity analyses

The results of significance tests for the GLM of the total number of ovulations (fertile and infertile eggs that were laid) of each female with the variables of weight and SVL show that neither of those variables are significantly related (Table 8). The box plots of SVL and weight, respectively, against the total number of ovulations show that there is seemingly no relation between the two variables (Figures 18 and 19).

Table 8 - Univariate Tests of Significance for GLM of total number of ovulations with the weight and SVL variables.
Sigma-restricted parameterization. Effective hypothesis decomposition; Std. Error of Estimate: 0.8489.

Effects	SS	df	MS	F	p
Intercept	1.09	1	1.09	1.51	0.22
Weight	1.24	1	1.24	1.73	0.19
SVL	1.38	1	1.38	1.92	0.17
Weight*SVL	1.46	1	1.46	2.03	0.16
Error	49.72	69	0.72		

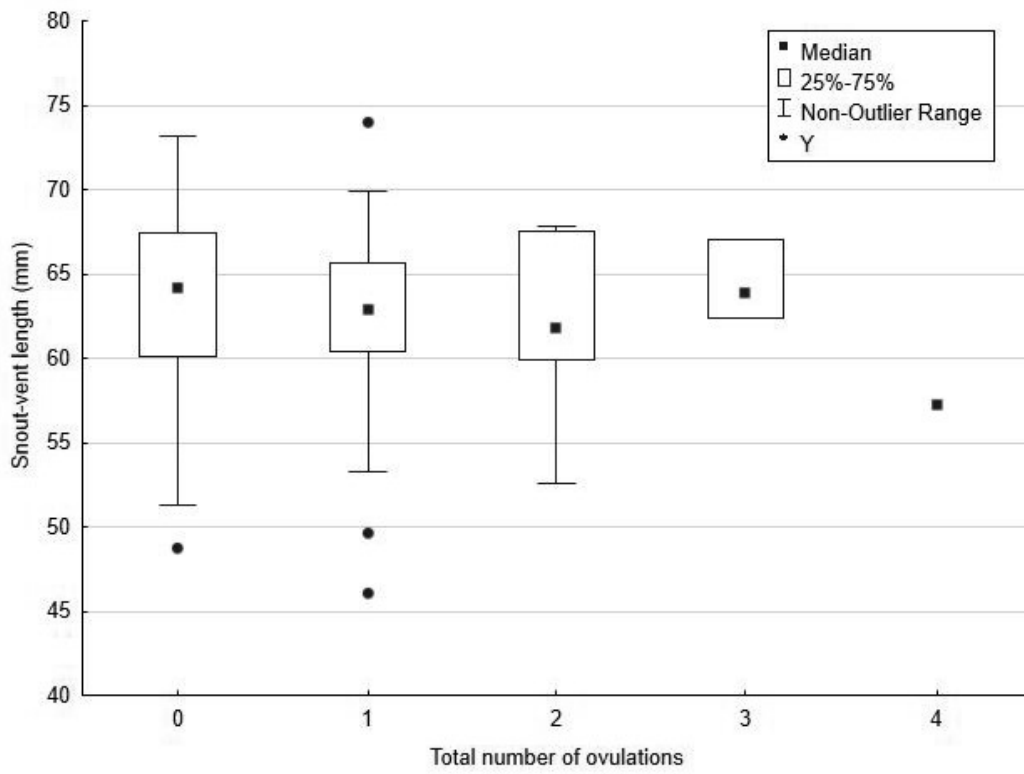


Figure 18 - Total number of ovulations by each differently sized female (in mm).

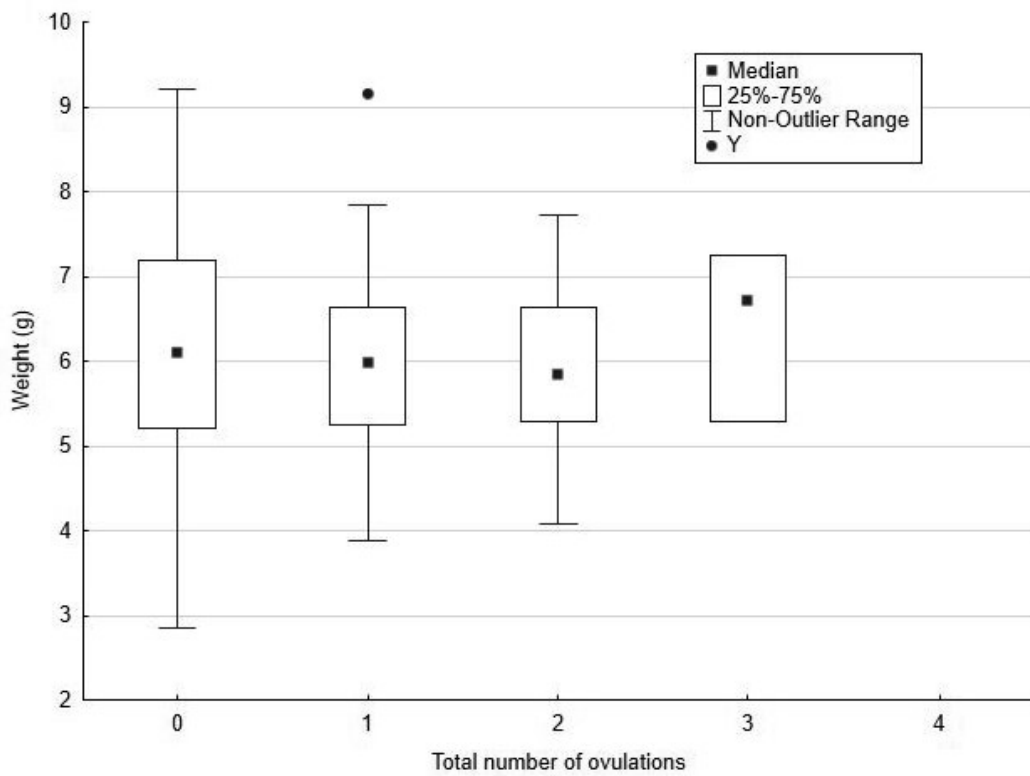


Figure 19 - Total number of ovulations by females with different weights (in g).

As for the clutch size and female weight, both the box plot and the p value of 0.98 from the Kruskal-Wallis test indicate that there is no statistically significant relation between these two variables (Figure 20).

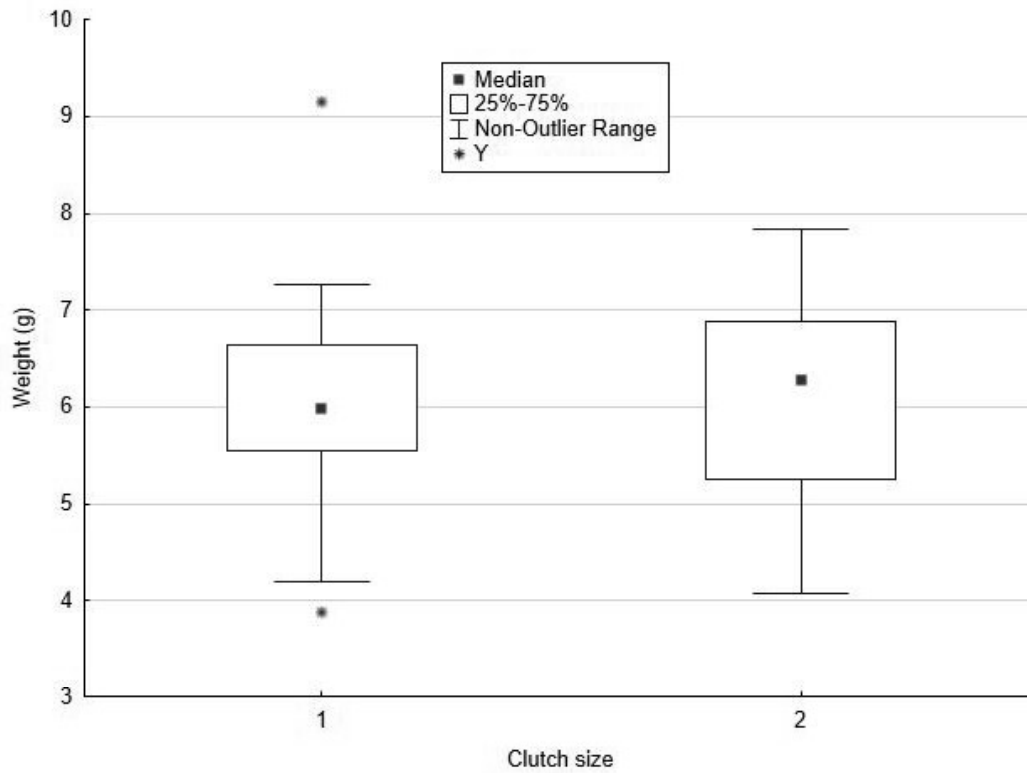


Figure 20 - Clutch size of females with different weights (in g).

4. Asymmetry

As shown in the histograms, adults from all populations present a zero-centered normal distribution due to FA, except in Évora, which is slightly skewed towards the right; adult geckos from Portimão are clearly more symmetrical than those from the rest of the populations (Figure 21). The results of a t-test indicate that there is, indeed, a statistically significant difference between this population and all the rest (Table 9). The histograms for FA for each adult sex within each population indicate very similar normal distributions for every population except from Évora, where females seem to be the ones skewing the distribution towards asymmetrical values (Figure 22). However, no statistically significant difference is found between both sexes of this population (Table 10).

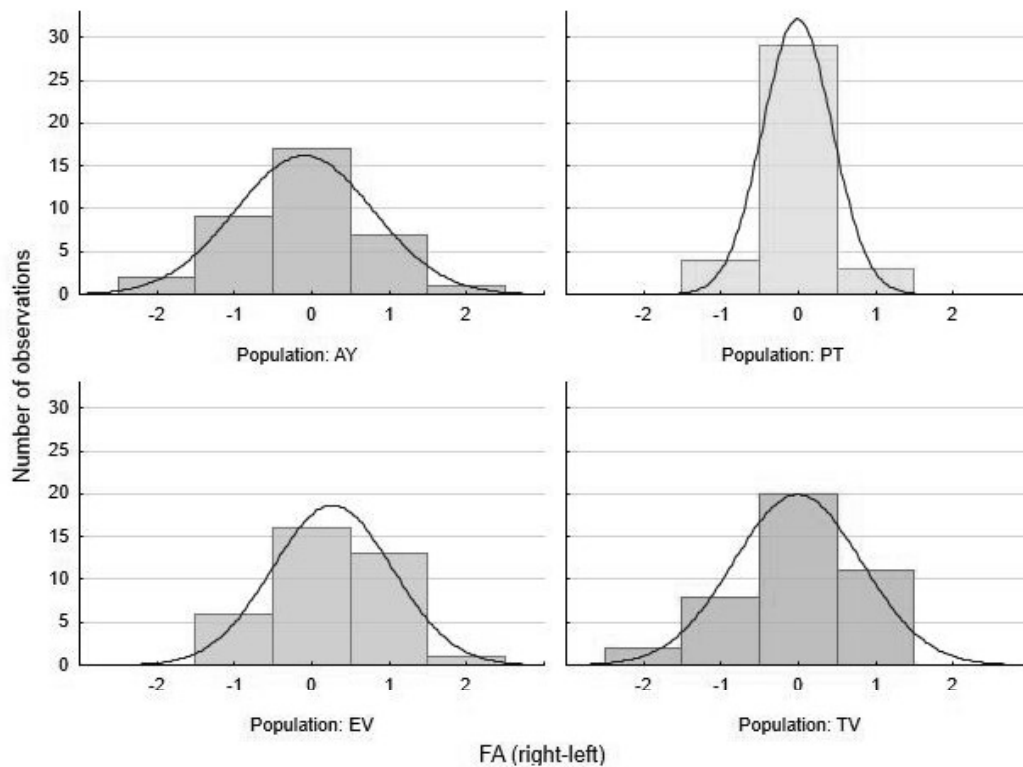


Figure 21 - Frequency of the different values of FA in adults, categorized by population.

Population: AY - FA = $36 \cdot 1 \cdot \text{Normal}(\text{Location}=-0.1111; \text{Scale}=0.8873)$;

Population: EV - FA = $36 \cdot 1 \cdot \text{Normal}(\text{Location}=0.25; \text{Scale}=0.77)$;

Population: PT - FA = $36 \cdot 1 \cdot \text{Normal}(\text{Location}=-0.0278; \text{Scale}=0.4463)$;

Population: TV - FA = $41 \cdot 1 \cdot \text{Normal}(\text{Location}=-0.0244; \text{Scale}=0.8212)$.

Table 9 - T-test results for the asymmetry between all populations.

Variables (1-2)	Mean Var. 1	Mean Var. 2	t-value	df	p-value	Std. Dev. Var. 1	Std. Dev. Var. 2	F-ratio Variances	p Variances
25-28	0.50	0.50	0.00	20	1.00	4	18	0.58	0.51
28-30	0.50	0.67	-0.69	22	0.50	18	6	0.51	0.52
25-30	0.50	0.67	-0.48	8	0.64	4	6	0.58	0.52

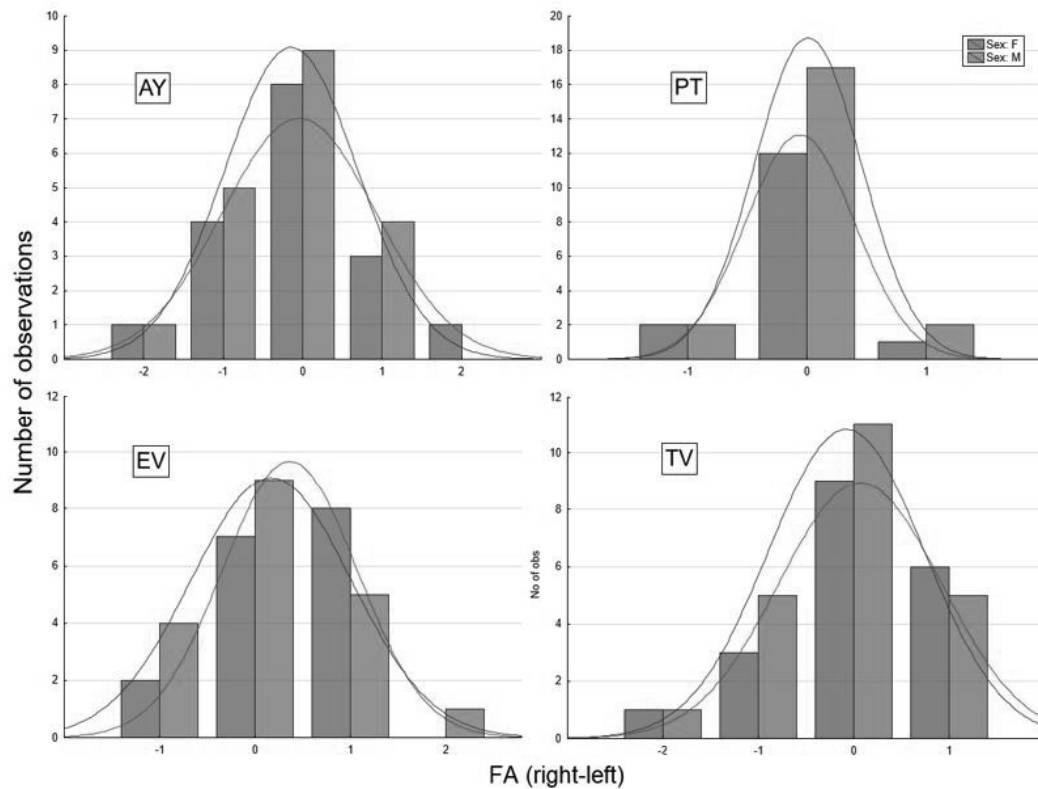


Figure 22 - Frequency of the different values of FA in adults, categorized by sex, within each population.

Sex: F - FA = 68*1*Normal(Location=0.0735; Scale=0.7788);
 Sex: M - FA = 81*1*Normal(Location=-0.0247; Scale=0.7412).

Table 10 - T-test results for the asymmetry between both sexes of each population.

Population	Mean F	Mean M	t-value	df	p-value	Std. Dev. F	Std. Dev. M	F-ratio Variances	p Variances
AY	0.529412	0.526316	0.018053	34	0.985702	17	19	0.514496	0.512989
PT	0.200000	0.190476	0.069181	34	0.945251	15	21	0.414039	0.402374
EV	0.588235	0.526316	0.363442	34	0.718525	17	19	0.507300	0.512989
TV	0.526316	0.500000	0.164007	39	0.870573	19	22	0.512989	0.511766

As for the FA in hatchlings, according to the results of the significance tests for GLM with the variables of adult female FA and incubation temperature, none of the variables are statistically significant (Table 11). A frequency scatter plot of hatchling FA against adult female FA shows that the two variables seem to not be related (Figure 23). The histograms for values of FA of hatchlings incubated at the different temperatures show that, even though there is lack of data at temperatures of 30°C and 25°C, the hatchlings incubated at 28°C seem to be more symmetrical than the rest (Figure 24).

Table 11 - Univariate Tests of Significance for GLM of hatchling FA with the female FA and incubation temperature variables. Sigma-restricted parameterization. Effective hypothesis decomposition; Std. Error of Estimate: 0.9517.

Effects	SS	df	MS	F	p
Intercept	0.04	1	0.04	0.049	0.83
Female FA	0.03	1	0.04	0.04	0.84
Incubation temperature	1.05	2	0.52	0.58	0.57
Error	15.40	17	0.90		

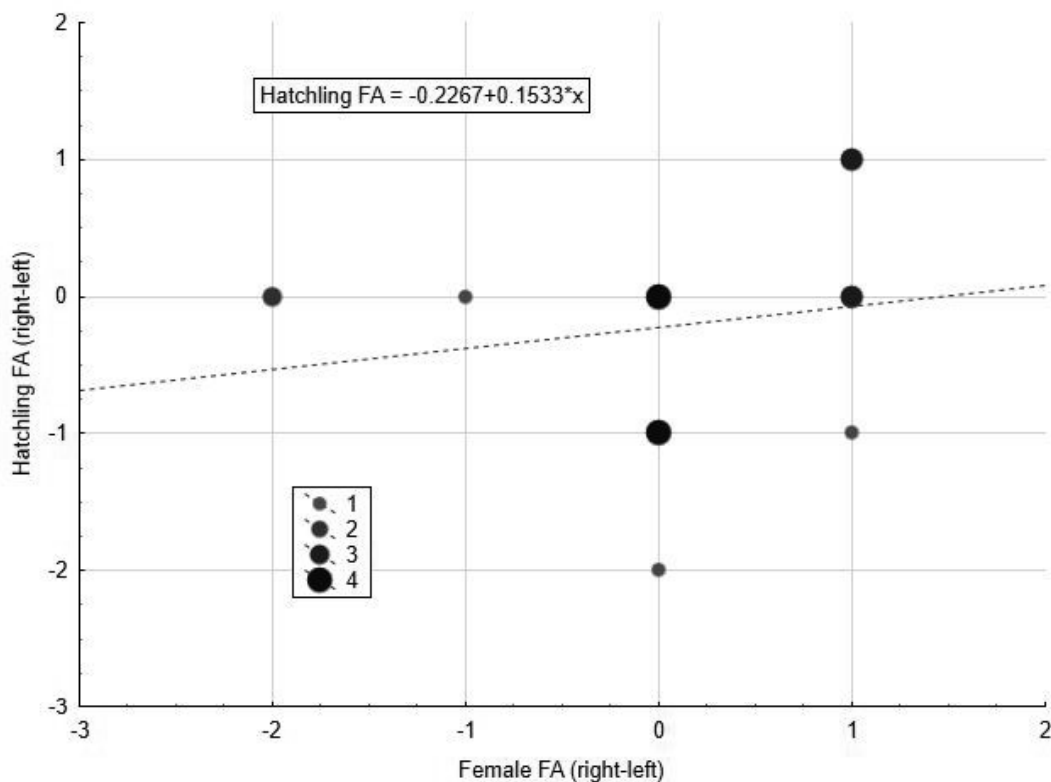


Figure 23 - Frequency of hatchling FA against female FA.

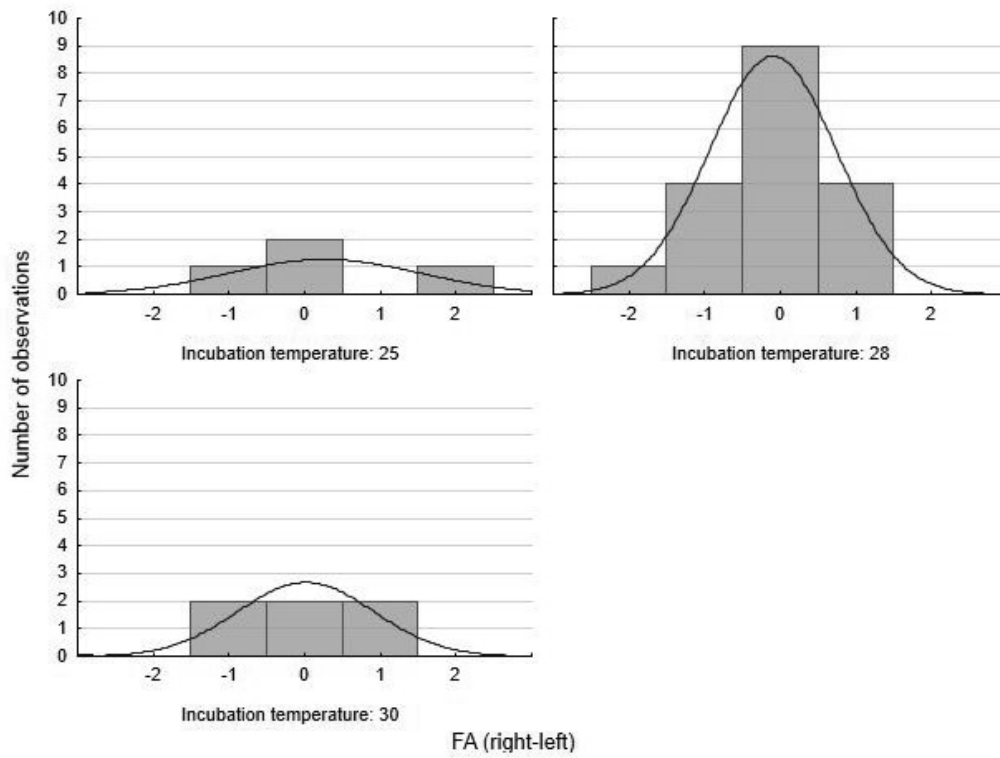


Figure 24 - Frequency of the different values of FA in hatchlings, categorized by incubation temperature.
 Incubation temperature: 30 - FA = 6*1*Normal(Location=2.7756E-17; Scale=0.8944);
 Incubation temperature: 28 - FA = 18*1*Normal(Location=-0.1111; Scale=0.8324);
 Incubation temperature: 25 - FA = 4*1*Normal(Location=0.25; Scale=1.2583).

Table 12 - T-test results for the asymmetry between hatchlings of different incubation temperatures.

Variables (1-2)	Mean Var. 1	Mean Var. 2	t-value	df	p-value	Std. Dev. Var. 1	Std. Dev. Var. 2	F-ratio Variances	p Variances
25-28	0.500000	0.500000	0.00	20	1.000000	4	18	0.577350	0.514496
28-30	0.500000	0.666667	-0.686607	22	0.499504	18	6	0.514496	0.516398
25-30	0.500000	0.666667	-0.478091	8	0.645376	4	6	0.577350	0.516398

5. Local thermal conditions

Average nest daily temperatures and daily thermal variance data obtained from the data loggers that were placed in predicted nesting spots in each population are presented in Figures 25 and 26. Unfortunately, due to construction work at the place where the dataloggers were placed in Évora, these were lost, so there is only information about the thermal conditions about the three remaining populations.

In Ayamonte, the mean daily temperatures rose significantly over the months of March through June, reaching the highest values in July and August, at times of 32°C, dropping quite abruptly in September (Figure 25). This locality experiences extremely high daily thermal variance, with values above 35°C at times (Figure 26). In comparison, both Portimão and Torres Vedras show lower temperatures and more stable temperatures, with much less thermal variance: in Portimão, the average daily temperatures reach their highest in July and August, but stay at around 24-26°C, never even going above 29°C and, again, dropping abruptly in the beginning of September; in Torres Vedras, the months of June and July experience the highest average daily temperature, mostly in between 22-24°C, rising and falling in a more gradual way in the preceding and following months (Figure 25 and 26).

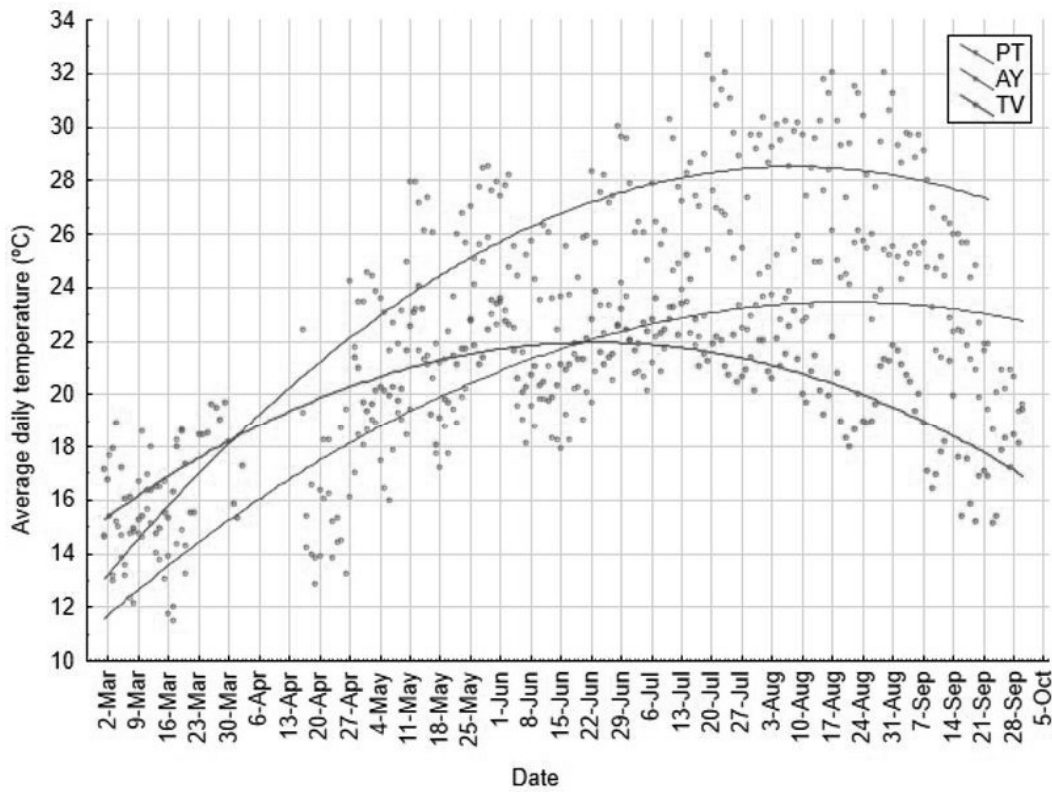


Figure 25 - Daily nest average temperature throughout the reproductive season in Ayamonte (blue), Portimão (green) and Torres Vedras (pink), with polynomial fittings.

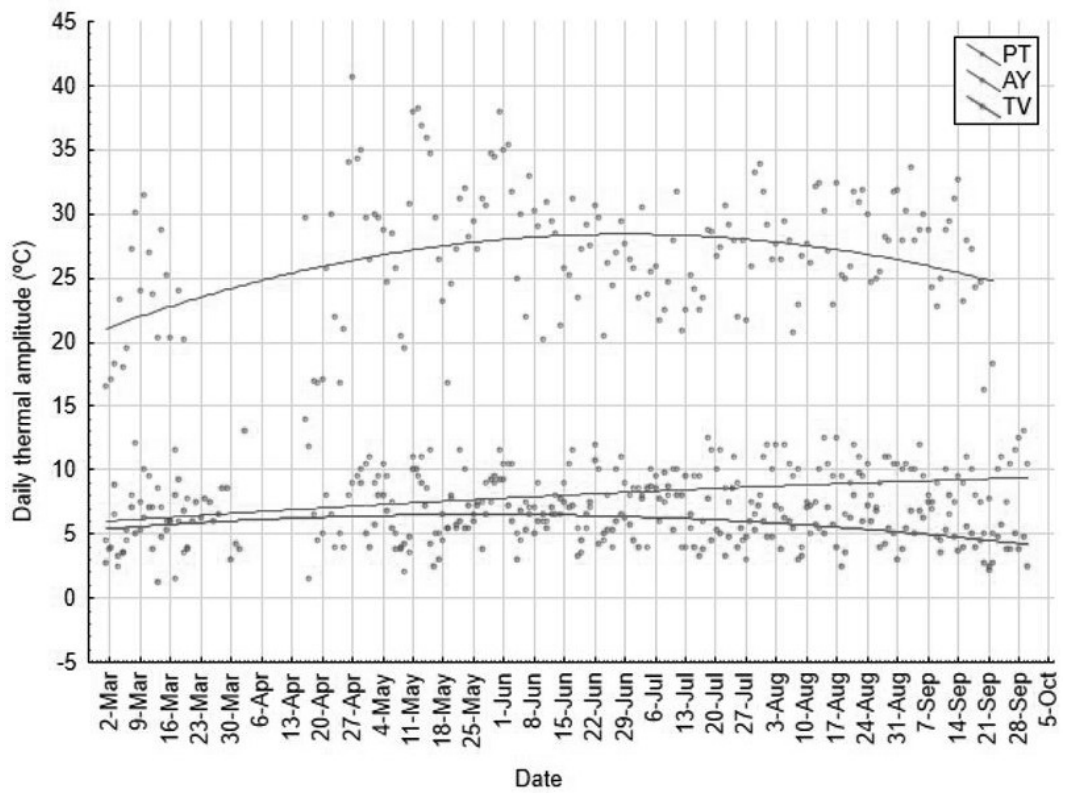


Figure 26 - Daily nest thermal variance throughout the reproductive season in Ayamonte (blue), Portimão (green) and Torres Vedras (pink), with polynomial fittings.

Discussion

The small sample size, result of the low fecundity of this species and the high mortality of the incubated hatchlings, is noticeable throughout most of the results and limits all possible inferences about the main objectives of this study. Although valid on its own for this dissertation project, the presented results are only a preliminary part of a main four-year research project. Nevertheless, in general, the obtained results suggest that the phenological traits of *Tarentola mauritanica* included in this study go in line with previous research done on phylogenetically related species. Even though in most of the cases our inferences are clearly limited by small sample sizes and/or logistic constraints, the thermal ecology of reproduction in this species seems to be mostly conservative and does not appear to be undergoing local selection.

1. Sex-ratio and pivotal temperature

The results on mortality (Figure 7) suggest that different incubation temperatures result in different hatchling viability rates, with the lowest mortality being recorded at 28°C and increasing in extreme temperatures. This hypothesis has not yet been explored in the past in this species and should be taken into account when performing any experimental studies on documented TSD species in the future, due to the phenomenon of sex-specific different mortalities potentially being mistaken for TSD (Wapstra *et al.* 2009; Göth & Booth, 2005). One way to avoid this confusion is to dissect non-viable embryos and check the sex; this is still pending some technical limitations in this study.

Looking at the sex-ratio at the different incubation temperatures, the distinction between the yield of female and male hatchlings at higher and lower incubation temperatures, respectively (Figure 8) goes in line with the hypothesis of a MF TSD pattern in *Tarentola mauritanica* (Hielen, 1992; Nettmann & Rykena, 1985). However, our results show a pivotal temperature at around 27.78°C (Figure 9), a difference of 0.98°C from the 26.8°C suggested by Hielen (1992). The abrupt TRT suggested by the regression line of Figure 9 is not surprising considering that there were only three male hatchlings and at the incubation temperature of 28°C the male-to-female hatchling ratio is very low. This is possibly warping the pivotal temperature, pulling it more towards higher values. However, if we come to discover further indications of a higher pivotal

temperature than that of Hielen (1992) and, considering that an incubation temperature of 28°C renders the least mortality, selection could be favouring a balanced sex-ratio. There is definitely the need for more data to be collected on colder, male-inducing incubation temperatures in the upcoming years of this research project, where more fine-tuned incubation tests will need to be done in order to infer more specific conclusions about the pivotal temperature and the TSP.

The clear negative correlation between incubation temperature and total incubation time (Figure 10), is well known in reptiles and goes in line with previous research that shows that the higher the incubation temperature, the faster the developmental rate (Richard & Shine, 1996; Du *et al.*, 2009).

In contrast with the results of Braña & Ji (2000) on *Podarcis muralis* and Deeming & Ferguson (1989) on *Alligator mississippiensis*, who indicate that faster developing eggs result in smaller hatchlings overall, incubation temperature does not significantly explain hatchling weight in *T. mauritanica* and there is seemingly no relationship between these two variables so far in this study (Figure 11). A different study done on *Anolis carolinensis* suggests that egg incubation temperature has latent effects on juvenile growth despite the absence of any detected immediate effects on hatchling phenotype (Goodman, 2008). Being that this species also lays two egg clutches, this suggests that, in K-strategists, temperature may modulate hatchling survival while in r-strategist it is not very relevant. Studies performed on bearded dragons, *Pogona vitticeps*, suggest that incubation temperature not only have an impact upon short term 'boldness', but also on the foraging behaviour of juvenile lizards, with a potential trade-off between growth and foraging speed, which could influence an animal's life history trajectory (Siviter *et al.*, 2016; Siviter *et al.*, 2019). More data needs to be collected and more in depth analyses need to be performed on the growth rate of hatchlings incubated at different temperatures in order to take any conclusions about incubation temperature affecting growth rate in *T. mauritanica*.

The finding that female (mother) weight is positively related to the hatchling weight (Table 2, Figure 12) is not surprising: heavier (bigger or fatter) females are, in theory, able to produce bigger eggs with more stored nutrients, which in turn would result in bigger and stronger hatchlings. This is a normal trend in the life history of organisms with very small offspring size, what is called K-selection - more energy is put into each individual offspring, increasing individual fitness, but decreasing fecundity - as opposed to r-selection - the production of many offspring, each with a relatively low

probability of surviving to adulthood (Pianka, 1970). However, as large as they may be, *T. mauritanica* females cannot lay more than two eggs, so they invest their extra resources in producing larger juveniles which will, naturally, have a higher survival rate after hatching (Sinervo, 1990). The positive relationship between these two variables was recorded in the leopard gecko, *Eublepharis macularius*, in Tousignant & Crews (1995).

2. Oviposition temperature choice

The clearly non-significant results of the tests with the population variable explaining oviposition temperature (Table 4, Figure 13) are in accordance with the first hypothesis of the expected results for this research objective: the lack of local adaptation of populations across the species' geographic range and, therefore, phylogenetic inertia. The possible inability of these animals to adjust nest-site choice with the predicted rapid climate shifts will have consequences in the long term for, at least, the populations included in this study. However, it is also worth noting that, as with all experimental studies, artificial lab conditions (although designed in order to emulate natural conditions as close as possible) and even the simple act of captivity during the reproductive period may have increased background noise, causing some females to lay eggs induced by stress and not by temperature preference, which increases variation in the results.

The variable that best described the oviposition temperature chosen by females was the oviposition date (Table 3), although not even this relationship was statistically significant. However, even though the need to collect more data is clear, considering the clearly non-significant results of other tests and the fact that the scatterplot of Figure 14 shows a small trend for a positive relation between these two variables, this suggests the possibility that slightly higher temperatures are chosen later in the reproductive season. Previous studies done on water dragons, *Intellagama lesueurii* (Pezaro *et al.*, 2016), and jacky dragons, *Amphibolurus muricatus* (Warner & Shine, 2011), indicate that higher mean nest temperatures are recorded later in the reproductive season for both species. This phenomenon does result in lower yields of females in earlier-laid clutches and higher in later-laid clutches (and vice-versa for males) but, due to a faster embryo growth rate at higher incubation temperatures (Figure 10), the mean hatching date for earlier and later laid clutches does not differ by

a lot (Pezaro *et al.*, 2016). It is unknown if these different oviposition temperatures/mean nest temperatures are purposefully chosen by females for an undisclosed increase in hatchling fitness or are just the result of an increase of mean temperatures throughout the reproductive season, such minor age differences between male and female hatchlings may not indicate sex-related fitness differences later in life (Pezaro *et al.*, 2016), as assumed for some models for the evolution and maintenance of TSD (Charnov & Bull, 1977; Janzen & Phillips, 2006). However, Harlow and Taylor (2000) suggests that increasing temperature during the nesting season correlates with increasing female offspring in a fast-growing, short-lived species (jacky dragon, *Amphibolurus muricatus*) and that male early hatchlings grow for a longer time, ending up larger than females, a trait that is probably favored by sexual selection.

Contrarily, when the relation between oviposition temperature and date was analysed per population, Ayamonte and Torres Vedras do not follow the general trend (Figure 15). There is an overall lack of data, which is obviously even more pronounced when analysing each individual population and, for example, in Ayamonte's case, this is due to a lack of representative data across their reproductive season; in this population, females stopped reproducing between June 14 and June 24, and so basically, we have around one month of data. In fact, according to González de la Vega (1988) the reproductive season of *T. mauritanica* from Huelva takes place between March and July. Hence, it is much sooner than what seems to occur with other populations. In the future, it is absolutely imperative to collect animals during March in order to obtain the full oviposition temperature data spectrum. Regarding the populations from Torres Vedras, apart from the fact that reproduction seems to start somewhat later, the temperature for oviposition along the season is more or less stable.

Nevertheless, if all eggs were incubated at the mean temperature selected by the females for oviposition, all embryos would develop as females. This indicates that male production is possibly the result of environmental constraints (climate, variation in substrate temperatures) rather than of a specific choice of the mothers. This interplay between female behaviour and environment might result in geographic variations in sex ratio at birth but not necessarily at adult stage. Still, one should also take into account the phenology of animals from different populations; when and where mating takes place and females lay their eggs.

3. Female fecundity analyses

The total number of ovulations (fertile and infertile eggs that were laid) of each female do not seem to be correlated with their weight or SVL and neither of those variables are statistically significant (Table 8, Figures 18 and 19). Two previous studies performed on metallic skinks, *Niveoscincus metallicus* (Jones & Swain, 1996) and Okinawa habus, *Trimeresurus flavoviridis* (Shiroma, 1993), suggest that the number of ovulated eggs or embryos/follicles or eggs on oviducts of mature females are positively correlated with SVL but, for these species, this possibly relates to the available space for eggs/embryos to develop. However, due to the invariant (or almost invariant) clutch size in *T. mauritanica*, compared to the variable clutch size of other species, the number of total clutches would probably be related not to the available space inside the female, but to the nutrients/calcium the female could spare for egg development. Geckos and other species that lay 1-2 egg clutches should only modulate reproductive effort through egg size and clutch frequency. Still, no relation between these two variables was found for our model species in this study so far. As for the frequency of 1/2 eggs clutches, although, in theory, larger females with more nutrients to spare for reproduction would lay 2 egg clutches more often and vice versa, our results show indicate that this isn't the case, at least in our model species and with the data collected until now (Figure 20).

4. Asymmetry and local thermal conditions

In relation to the level of FA, each population presents slightly different patterns, but all populations appear to be normally distributed around 0 apart from Évora, which is slightly biased towards asymmetrical values, being therefore closer to directional asymmetry; the biggest and only significant difference, however, is found in Portimão, which is a lot more symmetrical than the other three populations (Figure 21, Table 9). Considering FA as an indicator of developmental instability, this may indicate that this population does not experience conditions as unstable when compared to the others, namely large temperature fluctuations and/or an average departing from the optimal temperature for embryonic development, at the time of incubation. As for each sex within each population, all patterns are the same except that, in Évora, the females seem to be the reason for the skewed distribution towards asymmetrical values, but not

the males (Figure 22). However, no significant differences were found between each sex in any population (Table 10).

As for the FA levels in hatchlings, this particular trait does not seem to be heritable from the mothers, as seen in the non-significant results of Table 11 and in the frequency scatterplot of Figure 23. However, when comparing FA values of hatchlings incubated at different temperatures, although there are no significant results and there is very little data for 25 and 30°C, it seems that hatchlings incubated at 28°C are more symmetrical than those incubated at either of the remaining temperatures (Figure 24, Table 11). Interestingly enough, referring back to the mortality results that show that the incubation temperature of 28°C was the one that recorded the least mortality of all (Figure 7). Combining these findings, it seems that 28°C is an ideal temperature for development conditions. However, females seem to select higher temperatures for oviposition (Figure 13) which, in terms of hatchling viability, is counter-intuitive but suggest other factors (such as operational sex-ratio or constraints for hatchling growth before hibernation) may be involved.

Piecing together the results of FA and the thermal data collected with the data loggers, the reason for the apparent bigger symmetry in the population of Portimão and the asymmetrical pattern of females from Évora becomes unclear. Even though the data for the locality of Évora was lost, considering this is the innermost location, day and night temperatures should fluctuate a lot compared to the other populations, especially Portimão and Torres Vedras; these two populations present relatively low daily thermal variance and, on the other hand, Ayamonte, apart from experiencing the highest average daily temperatures of the three, presents a very high level of daily thermal variance (Figures 25 and 26). These observations would indicate that, comparing only these three populations, Ayamonte and possibly Évora would be the least symmetrical populations, due to the daily big fluctuations in temperature that embryos would undergo when incubating. However, this is not the case and no relation between local thermal conditions and FA patterns is found.

Conclusion

The results obtained so far in this study under very strict experimental conditions, provide confirmation of preliminary studies suggesting TSD in *Tarentola mauritanica* and, specifically, an MF pattern. They also indicate that the thermal ecology of reproduction in this species is mostly conservative with no evidence of local adaptation of female nest-site choice, a key TSD component. Due to limited sample size, pivotal temperature could not be compared among populations, but will be so in the following years of this project. Apparently, different FA patterns seem to be present among populations. However, no connection to local incubation temperature conditions was found and no heritability of this trait was proven. The sample size limitations noticed throughout the whole study should be emphasized because, along with logistic constraints, restricted the capability to attain part of the objectives of this dissertation project. Nevertheless, these results on this limited sample also indicate that the working methodology is valid and applicable on a larger scale in the near future. Overall, the premise of this study and the results presented so far opens promising research avenues on the functional analysis of the effects on environmental shifts on TSD species and, hence, on the repercussions of climate change and its minimizing measures.

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Appendix

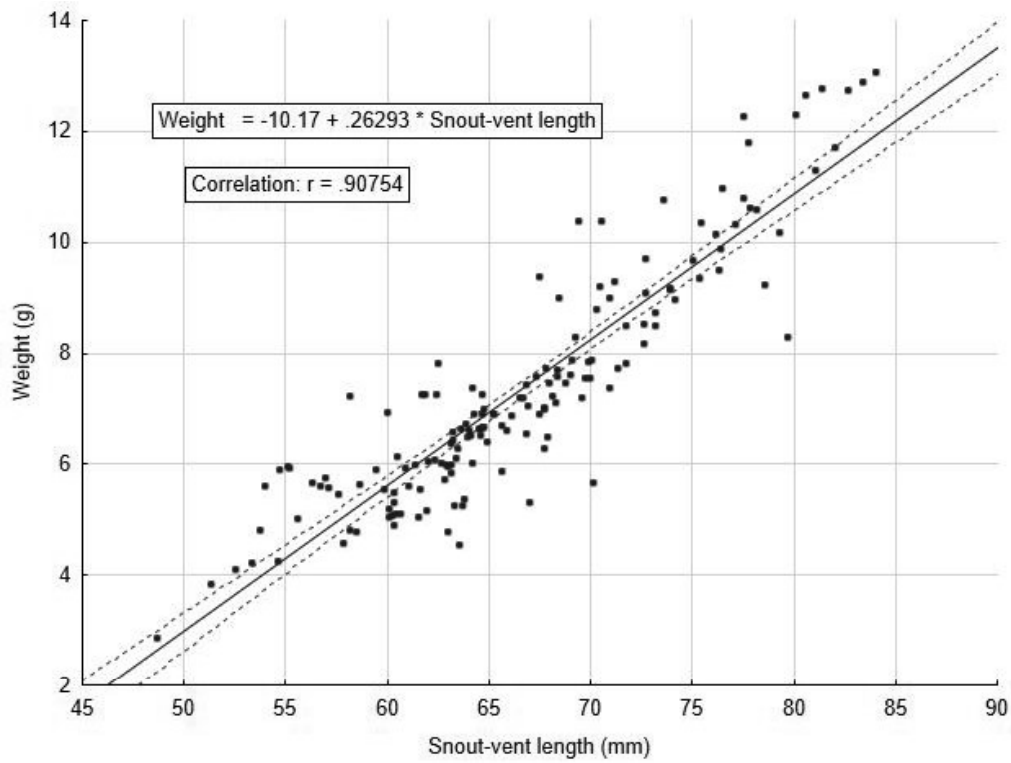


Figure 27 - Adult weight (in g) against SVL (in mm).

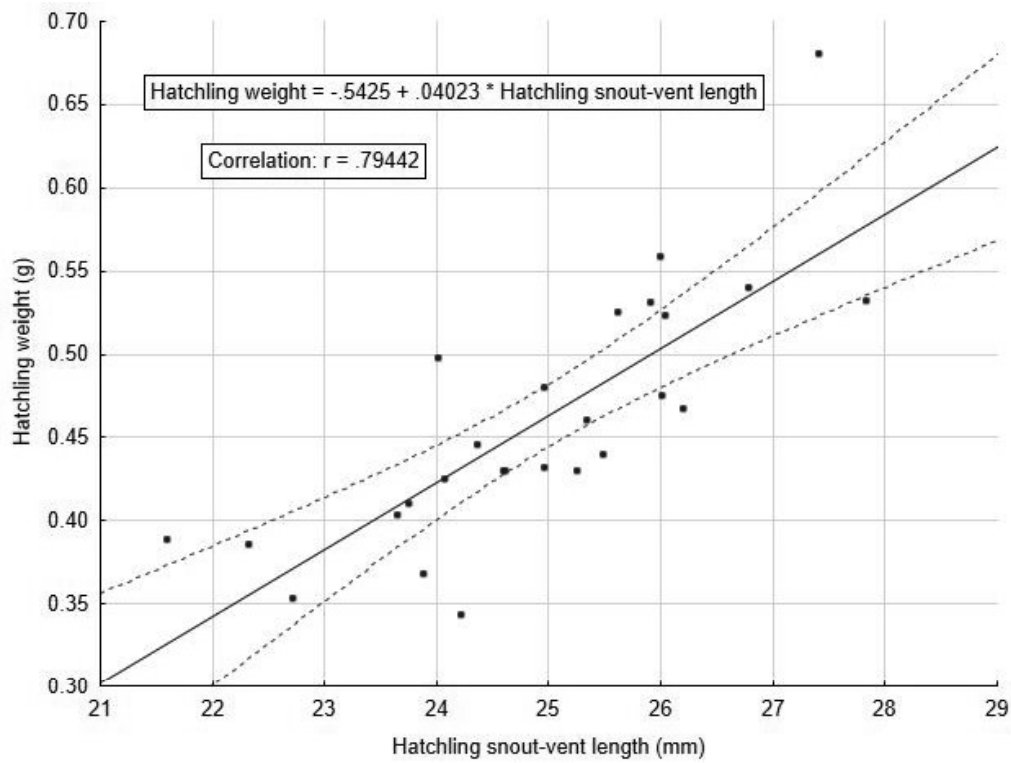


Figure 28 - Hatchling weight (in g) against hatchling SVL (in mm).

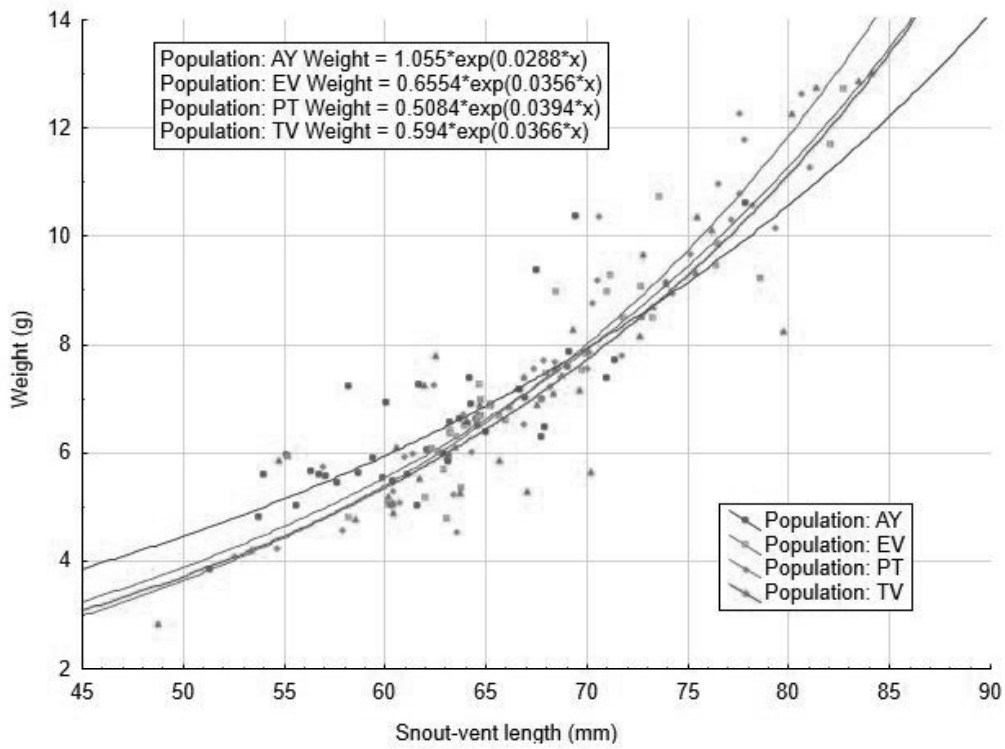


Figure 29 - Adult weight (in g) against adult SVL (in mm), categorized by population.

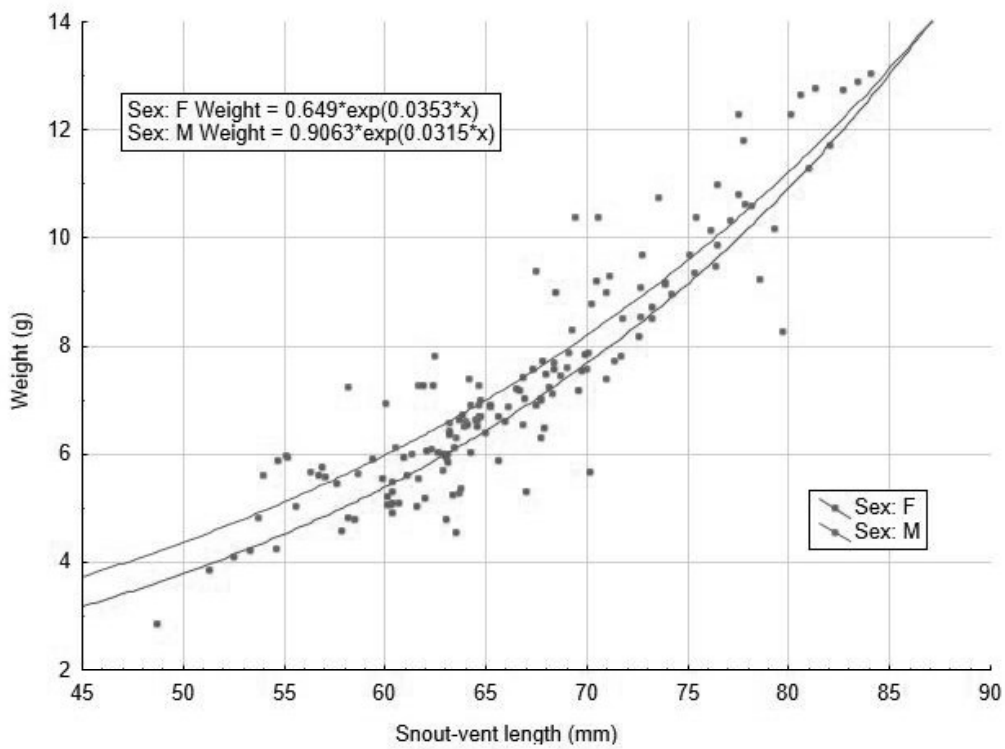


Figure 30 - Adult weight (in g) against SVL (in mm), categorized by sex, with pooled populations.